PATENT COOPERATION TREATY

PCT

09/509779

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
5650-01-MJA		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/ 26705	15/12/1998	19/12/1997
Applicant		
WARNER-LAMBERT COMPANY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of Invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/26705

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark n Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 25 and 26 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Insofar as claims 30, 35-37 may be said to relate to methods in vivo, i.e methods of treatment of the human/animal body, then objection arises under Art 17.2.a.1 PCT, therefore a search has been carried out partially and based on the alleged effects of the compound/composition.

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

Line 8: after "12.7 kDa." delete to "Furthermore" in line 14;
Line 15: start with the word "Antisense".

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/26705

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C12N15/12 A61K38/00 C07K16/18 C12Q1/68
C12N15/10 //C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L. ET AL.: "Soares parathyroid tumor NbHPA Homo sapiens cDNA clone 304814 5' " EMBL DATABASE,17 May 1996 (1996-05-17), XP002106007 HEIDELBERG, DE Accession Number: W38711 ---	1-5, 7-11, 18-21, 25,26
X	MARRA M. ET AL.: "Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 401232 5' " EMBL DATABASE,18 July 1996 (1996-07-18), XP002106008 HEIDELBERG, DE Accession Number: W98093 ---	1-5, 7-11, 18-21, 25,26
	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1999	20/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	DUAN H. ET AL.: "SAG, a novel zinc RING finger protein that protects cells from apoptosis induced by redox agents" MOLECULAR AND CELLULAR BIOLOGY, vol. 19, no. 4, 1999, pages 3145-3155, XP002106009 US the whole document ---	1-26,29, 30,32-35
X	SUN Y.: "Induction of glutathione synthetase by 1,10-phenantroline" FEBS LETTERS, vol. 408, no. 1, 1997, pages 16-20, XP002106010 AMSTERDAM NL cited in the application abstract ---	29
X	SUN Y. ET AL.: "Activation of p53 transcriptional activity by 1,10-phenanthroline, a metal chelator and redox sensitive compound" ONCOGENE, vol. 14, no. 4, 1997, pages 385-393, XP002106011 cited in the application abstract -----	29

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/47, C12N 15/12, A61K 38/00, C07K 16/18, C12Q 1/68, C12N 15/10 // C12N 15/82

A3

(11) International Publication Number: WO 99/32514

(43) International Publication Date: 1 July 1999 (01.07.99)

(21) International Application Number: PCT/US98/26705

(22) International Filing Date: 15 December 1998 (15.12.98)

(30) Priority Data:

60/068,179	19 December 1997 (19.12.97)	US
60/099,840	11 September 1998 (11.09.98)	US

(71) Applicant *(for all designated States except US)*: WARNER–LAMBERT COMPANY [US/US]; 201 Tabor Road, Morris Plains, NJ 07950 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: SUN, Yi [US/US]; 4841 Hillway Court, Ann Arbor, MI 48105 (US).

(74) Agents: RYAN, M., Andrea; Warner–Lambert Company, 201 Tabor Road, Morris Plains, NJ 07950 (US) et al.

(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HR, HU, ID, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
10 September 1999 (10.09.99)

(54) Title: SAG: SENSITIVE TO APOPTOSIS GENE

(57) Abstract

The present invention provides novel genes and polypeptides derived therefrom encoding a redox-sensitive protein that promotes cell growth, protects cells from apoptosis, scavenges oxygen radicals and can be used for the reversion of a tumor phenotype. In an attempt to identify gene(s) responsible for 1,10-phenanthroline (OP)-induced apoptosis in tumor cells we have used the differential display technique and cloned an OP-inducible gene, SAG (Sensitive to Apoptosis Gene). SAG encodes a novel, redox-sensitive, heme-binding protein with a zinc RING finger domain. The SAG protein consists of 113 amino acids with a calculated molecular weight of 12.7 kDa. Antisense SAG transfection inhibits certain tumor cell phenotypes in DLD1 human cell line and microinjection of SAG RNA stimulates cell growth. We propose that SAG protein is a cellular protective molecule functioning as a redox sensor to buffer oxidative-stress induced damage as well as a growth factor to stimulate cell growth. SAG protein will be an ideal molecular target in the development of drugs against neurodegenerative disorders, cancers, muscle dystrophy, and promoting wound healing.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 98/26705

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C12N15/12 A61K38/00 C07K16/18 C12Q1/68
C12N15/10 //C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	MARRA M. ET AL.: "Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 401232 5' " EMBL DATABASE,18 July 1996 (1996-07-18), XP002106008 HEIDELBERG, DE Accession Number: W98093 --- -/--	1-5, 7-11, 18-21, 25,26

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1999	20/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 98/26705

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	DUAN H. ET AL.: "SAG, a novel zinc RING finger protein that protects cells from apoptosis induced by redox agents" MOLECULAR AND CELLULAR BIOLOGY, vol. 19, no. 4, 1999, pages 3145-3155, XP002106009 US the whole document ---	1-26,29, 30,32-35
X	SUN Y.: "Induction of glutathione synthetase by 1,10-phenantroline" FEBS LETTERS, vol. 408, no. 1, 1997, pages 16-20, XP002106010 AMSTERDAM NL cited in the application abstract ---	29
X	SUN Y. ET AL.: "Activation of p53 transcriptional activity by 1,10-phenanthroline, a metal chelator and redox sensitive compound" ONCOGENE, vol. 14, no. 4, 1997, pages 385-393, XP002106011 cited in the application abstract -----	29

4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/26705

Box I Observations wher c rtain claims were found uns archabl (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 25 and 26 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Insofar as claims 30, 35-37 may be said to relate to methods in vivo, i.e methods of treatment of the human/animal body, then objection arises under Art 17.2.a.1 PCT, therefore a search has been carried out partially and based on the alleged effects of the compound/composition.

PATENT COOPERATION TREATY

5650

09/509778 PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY - Schreiben an Rose Armstrong

PCT

To:

MANSMANN, Ivo et al.
WARNER-LAMBERT COMPANY
Legal Division, Patent Department
c/o Gödecke AG
Mooswaldallee 1
D-79090 Freiburg
ALLEMAGNE

Patentwesen
Eing.: 18. Jan. 2000

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	14. 01. 00
Applicant's or agent's file reference PD-5650-01-MJA	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/26705	15/12/1998	19/12/1997

Applicant
WARNER-LAMBERT COMPANY et al.

Express Mail No. EJ676053129US
PD-5650-01-MJA

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international applicatior

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PD-5650-01-MJA		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/26705	15/12/1998	19/12/1997
International Patent Classification (IPC) or national classification and IPC C07K14/47		
Applicant WARNER-LAMBERT COMPANY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08/07/1999	14.01.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Giebeler, K Telephone No. +49 89 2399 8546

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/26705

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-41 as originally filed

Claims, No.:

1-37 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6,12-14.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☒ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	15-17,22,24-28,30-37
	No:	Claims	1-5,7-11,18-21,23,29
Inventive step (IS)	Yes:	Claims	15-17,22
	No:	Claims	24-28,30-37
Industrial applicability (IA)	Yes:	Claims	1-5,7-11,15-29,32-34,37
	No:	Claims	30,31,35,36

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. It appears that the present description does not contain any information about the nature of the deposited cells of claims 6 and 12-14. Said claims thus lack clarity and support by the description as well as enablement. Therefore, no meaningful opinion on the subject-matter of said claims could be formed.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

2. The following documents are cited:

   D1: HILLIER L. ET AL.: 'Soares parathyroid tumor NbHPA Homo sapiens cDNA clone 304814 5' ' EMBL DATABASE, 17 May 1996 (1996-05-17), XP002106007 HEIDELBERG, DE

   D2: MARRA M. ET AL.: 'Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 401232 5' ' EMBL DATABASE,18 July 1996 (1996-07-18), XP002106008 HEIDELBERG, DE

   D3: SUN Y., FEBS LETTERS, vol. 408, no. 1, 1997, pages 16-20

   D4: SUN Y. ET AL., ONCOGENE, vol. 14, no. 4, 1997, pages 385-393

3. The present application does not satisfy the criterion set forth in Article 33(1)(2) PCT because the subject-matter of claims 1-5, 7-11, 18-21, 23, 29 is not new.

   The document D1 discloses a DNA which is 98.1% identical to the one shown in SEQ ID NO: 3 and therefore prejudicial to the novelty of claims 1-4, 7-10. The novelty of these claims is also affected by D2 which discloses a DNA 99.1% identical to the one of SEQ ID NO: 3.

   The method of claim 29 was already known from the art, see either of the documents D3 or D4.

Further novelty objections arise from the fact that claims 1, 3, 7, 9 and 18-21 do not clearly define the claimed subject-matter. The **unclear terms** "substantially similar" and "essentially" **cannot be used to distinguish the claimed matter from the prior art**. According to the description, page 5, lines 13-15, the term "substantially similar" includes "deletion, substitution or additions to a DNA, RNA or protein sequence that maintain the function of the protein product and possess similar zinc-binding motifs". While there are thus no limitations as to the sequence the claimed product, it is furthermore not defined which function(s) of the protein should be maintained. The protein according to the application possesses several functions which it shares with a lot of other proteins known from the prior art, for instance redox sensitivity, RNA-binding or ability to be induced by OP. The prior art products would thus appear to be encompassed by said claims, especially since the term "similar zinc-binding motifs" is also unclear and therefore not suitable to distinguish the claimed subject-matter from the prior art.
Furthermore, it would appear that the known yeast and C. elegans genes referred to on page 15, lines 24-27 of the description would hybridize to the genes of the application under the conditions defined on page 5, last paragraph.
Therefore, at least claims 1-5, 7-11, 18-21 and 23 are considered to lack novelty.

4. However, the specific sequences of claims 15 and 22 appear to be both novel and inventive over the prior art.
Claims 24-28 and 30-36 would only be considered as inventive if these claims clearly referred to the SAG protein having the sequences of SEQ ID NO: 2, 4 or those of claim 22, or to the DNA of SEQ ID NO: 1, 3 or those of claim 15.
However, no inventive step can be acknowledged for these claims in so far as they refer to known molecules (see point 3. above).

5. For the assessment of the present claims 30, 31, 35 and 36 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

6. The application does not meet the requirements of Article 6 PCT because claims 1, 3-5, 7, 9-11, 16-21, 23, 25-28 and 32 are not clear.

The term "substantially similar" used in claims 1, 7, 18, 20 and 23 and the term "consists essentially" used in claims 3, 9, 19 and 21 are vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which they refer, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT), see also point 3 above.
Claim 23 is particularly unclear since by reference to claims 18-22, it defines a polypeptide which is "substantially similar" to a polypeptide that is "substantially similar to" or "consist essentially of" a defined amino acid sequence.

The scope of claims 4, 10 and 16 is also unclear. Although it would appear that these claims should be directed to an extrachromosomal vector containing the DNA in question, this is not entirely clear from the wording of the claims.

Claims 5, 11 and 17 are directed to a "host cell comprising a host cell". These claims could be interpreted as being directed to those kind of cells that may incorporate other cells, for instance macrophages. However, there is no support in the description for such cells.

Claims 25 and 26 refer to primers "derived" from the DNA sequences of claims 1-3, 7-9 or 15. In principle, practically **any** DNA sequence can be "derived" from a given DNA sequence by making substitutions. The term "derived" is therefore completely unclear.

The term "SAG" was not known at the priority date of the present application and was therefore unclear to persons skilled in the art. Although page 2, lines 25-29 of the description defined the term "SAG protein" as having the amino acid sequence of SEQ ID NO: 2 or 4, this definition should be given in claims 25-28 and 32. Alternatively, a reference to a claim directed to the protein could be introduced.

7. Although claims 1, 2 and 3 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other only with regard to the definition of the subject-matter for which protection is sought. The aforementioned claims therefore lack conciseness. Moreover, lack of clarity of the claims as a whole arises, since the plurality of independent claims makes it difficult, if not impossible, to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of the protection.
   Hence, claims 1, 2 and 3 do not meet the requirements of Article 6 PCT.
   The same applies to claims 7, 8 and 9, claims 18 and 19, and claims 20 and 21.

8. Claim 37 which relates to the promotion or inhibition of the growth of plant cells is neither supported by the description (Article 6 PCT), nor enabled by the present application (Article 5 PCT).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PD-5650-01-MJA		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/26705	15/12/1998	19/12/1997
International Patent Classification (IPC) or national classification and IPC		
C07K14/47		
Applicant		
WARNER-LAMBERT COMPANY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which hav been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08/07/1999	14.01.00
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Giebeler, K Telephone No. +49 89 2399 8546

Form PCT/IPEA/409 (cover sheet) (January 1994)

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-41 as originally filed

Claims, No.:

1-37 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6,12-14.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☒ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	15-17,22,24-28,30-37
	No:	Claims	1-5,7-11,18-21,23,29
Inventive step (IS)	Yes:	Claims	15-17,22
	No:	Claims	24-28,30-37
Industrial applicability (IA)	Yes:	Claims	1-5,7-11,15-29,32-34,37
	No:	Claims	30,31,35,36

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. It appears that the present description does not contain any information about the nature of the deposited cells of claims 6 and 12-14. Said claims thus lack clarity and support by the description as well as enablement. Therefore, no meaningful opinion on the subject-matter of said claims could be formed.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

2. The following documents are cited:

   D1: HILLIER L. ET AL.: 'Soares parathyroid tumor NbHPA Homo sapiens cDNA clone 304814 5' ' EMBL DATABASE, 17 May 1996 (1996-05-17), XP002106007 HEIDELBERG, DE

   D2: MARRA M. ET AL.: 'Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 401232 5' ' EMBL DATABASE,18 July 1996 (1996-07-18), XP002106008 HEIDELBERG, DE

   D3: SUN Y., FEBS LETTERS, vol. 408, no. 1, 1997, pages 16-20

   D4: SUN Y. ET AL., ONCOGENE, vol. 14, no. 4, 1997, pages 385-393

3. The present application does not satisfy the criterion set forth in Article 33(1)(2) PCT because the subject-matter of claims 1-5, 7-11, 18-21, 23, 29 is not new.

   The document D1 discloses a DNA which is 98.1% identical to the one shown in SEQ ID NO: 3 and therefore prejudicial to the novelty of claims 1-4, 7-10. The novelty of these claims is also affected by D2 which discloses a DNA 99.1% identical to the one of SEQ ID NO: 3.

   The method of claim 29 was already known from the art, see either of the documents D3 or D4.

Further novelty objections arise from the fact that claims 1, 3, 7, 9 and 18-21 do not clearly define the claimed subject-matter. The **unclear terms** "substantially similar" and "essentially" **cannot be used to distinguish the claimed matter from the prior art**. According to the description, page 5, lines 13-15, the term "substantially similar" includes "deletion, substitution or additions to a DNA, RNA or protein sequence that maintain the function of the protein product and possess similar zinc-binding motifs". While there are thus no limitations as to the sequence the claimed product, it is furthermore not defined which function(s) of the protein should be maintained. The protein according to the application possesses several functions which it shares with a lot of other proteins known from the prior art, for instance redox sensitivity, RNA-binding or ability to be induced by OP. The prior art products would thus appear to be encompassed by said claims, especially since the term "similar zinc-binding motifs" is also unclear and therefore not suitable to distinguish the claimed subject-matter from the prior art.
Furthermore, it would appear that the known yeast and C. elegans genes referred to on page 15, lines 24-27 of the description would hybridize to the genes of the application under the conditions defined on page 5, last paragraph.
Therefore, at least claims 1-5, 7-11, 18-21 and 23 are considered to lack novelty.

4. However, the specific sequences of claims 15 and 22 appear to be both novel and inventive over the prior art.
Claims 24-28 and 30-36 would only be considered as inventive if these claims clearly referred to the SAG protein having the sequences of SEQ ID NO: 2, 4 or those of claim 22, or to the DNA of SEQ ID NO: 1, 3 or those of claim 15.
However, no inventive step can be acknowledged for these claims in so far as they refer to known molecules (see point 3. above).

5. For the assessment of the present claims 30, 31, 35 and 36 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

6. The application does not meet the requirements of Article 6 PCT because claims 1, 3-5, 7, 9-11, 16-21, 23, 25-28 and 32 are not clear.

The term "substantially similar" used in claims 1, 7, 18, 20 and 23 and the term "consists essentially" used in claims 3, 9, 19 and 21 are vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which they refer, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT), see also point 3 above.
Claim 23 is particularly unclear since by reference to claims 18-22, it defines a polypeptide which is "substantially similar" to a polypeptide that is "substantially similar to" or "consist essentially of" a defined amino acid sequence.

The scope of claims 4, 10 and 16 is also unclear. Although it would appear that these claims should be directed to an extrachromosomal vector containing the DNA in question, this is not entirely clear from the wording of the claims.

Claims 5, 11 and 17 are directed to a "host cell comprising a host cell". These claims could be interpreted as being directed to those kind of cells that may incorporate other cells, for instance macrophages. However, there is no support in the description for such cells.

Claims 25 and 26 refer to primers "derived" from the DNA sequences of claims 1-3, 7-9 or 15. In principle, practically **any** DNA sequence can be "derived" from a given DNA sequence by making substitutions. The term "derived" is therefore completely unclear.

The term "SAG" was not known at the priority date of the present application and was therefore unclear to persons skilled in the art. Although page 2, lines 25-29 of the description defined the term "SAG protein" as having the amino acid sequence of SEQ ID NO: 2 or 4, this definition should be given in claims 25-28 and 32. Alternatively, a reference to a claim directed to the protein could be introduced.

7. Although claims 1, 2 and 3 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other only with regard to the definition of the subject-matter for which protection is sought. The aforementioned claims therefore lack conciseness. Moreover, lack of clarity of the claims as a whole arises, since the plurality of independent claims makes it difficult, if not impossible, to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of the protection.
   Hence, claims 1, 2 and 3 do not meet the requirements of Article 6 PCT.
   The same applies to claims 7, 8 and 9, claims 18 and 19, and claims 20 and 21.

8. Claim 37 which relates to the promotion or inhibition of the growth of plant cells is neither supported by the description (Article 6 PCT), nor enabled by the present application (Article 5 PCT).

PATENT COOPERATION TREATY

09/509779

PCT

From the INTERNATIONAL SEARCHING AUTHORITY

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
MP PATENT DEPT
WARNER-LAMBERT COMPANY
Attn. RYAN, A.
201 Tabor Road
Morris Plains, New Jersey 07950
UNITED STATES OF AMERICA

Applicant's or agent's file reference 5650-01-MJA	Date of mailing *(day/month/year)* 20/07/1999
International application No. PCT/US 98/ 26705	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
Applicant WARNER-LAMBERT COMPANY et al.	International filing date *(day/month/year)* 15/12/1998

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV. Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Renate Jordan

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
5650-01-MJA		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/ 26705	15/12/1998	19/12/1997
Applicant		
WARNER-LAMBERT COMPANY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/26705

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 25 and 26 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Insofar as claims 30, 35-37 may be said to relate to methods in vivo, i.e methods of treatment of the human/animal body, then objection arises under Art 17.2.a.1 PCT, therefore a search has been carried out partially and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 26705

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first she t)

Line 8: after "12.7 kDa." delete to "Furthermore" in line 14;
Line 15: start with the word "Antisense".

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/26705

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C12N15/12 A61K38/00 C07K16/18 C12Q1/68
C12N15/10 //C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L. ET AL.: "Soares parathyroid tumor NbHPA Homo sapiens cDNA clone 304814 5' " EMBL DATABASE,17 May 1996 (1996-05-17), XP002106007 HEIDELBERG, DE Accession Number: W38711 ---	1-5, 7-11, 18-21, 25,26
X	MARRA M. ET AL.: "Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 401232 5' " EMBL DATABASE,18 July 1996 (1996-07-18), XP002106008 HEIDELBERG, DE Accession Number: W98093 --- -/--	1-5, 7-11, 18-21, 25,26

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/26705

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	DUAN H. ET AL.: "SAG, a novel zinc RING finger protein that protects cells from apoptosis induced by redox agents" MOLECULAR AND CELLULAR BIOLOGY, vol. 19, no. 4, 1999, pages 3145-3155, XP002106009 US the whole document ---	1-26,29, 30,32-35
X	SUN Y.: "Induction of glutathione synthetase by 1,10-phenantroline" FEBS LETTERS, vol. 408, no. 1, 1997, pages 16-20, XP002106010 AMSTERDAM NL cited in the application abstract ---	29
X	SUN Y. ET AL.: "Activation of p53 transcriptional activity by 1,10-phenanthroline, a metal chelator and redox sensitive compound" ONCOGENE, vol. 14, no. 4, 1997, pages 385-393, XP002106011 cited in the application abstract -----	29

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(54) Title: SAG: SENSITIVE TO APOPTOSIS GENE

(57) Abstract

The present invention provides novel genes and polypeptides derived therefrom encoding a redox–sensitive protein that promotes cell growth, protects cells from apoptosis, scavenges oxygen radicals and can be used for the reversion of a tumor phenotype. In an attempt to identify gene(s) responsible for 1,10–phenanthroline (OP)–induced apoptosis in tumor cells we have used the differential display technique and cloned an OP–inducible gene, SAG (Sensitive to Apoptosis Gene). SAG encodes a novel, redox–sensitive, heme–binding protein with a zinc RING finger domain. The SAG protein consists of 113 amino acids with a calculated molecular weight of 12.7 kDa. Sequence homology searches reveal that SAG is highly conserved among species, suggesting its functional importance. This suggestion is demonstrated by the finding that SAG disruption in yeast is lethal. Two SAG deletion mutants have been detected in human cancer cell lines originating from colon and testis, suggesting its possible role in human carcinogenesis. Overexpression of SAG protein in a human colon carcinoma line, DLD1, and a human neuroblastoma line, SY5Y, protects cells from apoptosis induced by OP, zinc and copper ions. Furthermore, antisense SAG transfection inhibits certain tumor cell phenotypes in DLD1 human cell line and microinjection of SAG RNA stimulates cell growth. We propose that SAG protein is a cellular protective molecule functioning as a redox sensor to buffer oxidative–stress induced damage as well as a growth factor to stimulate cell growth. SAG protein will be an ideal molecular target in the development of drugs against neurodegenerative disorders, cancers, muscle dystrophy, and promoting wound healing.

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SAG: SENSITIVE TO APOPTOSIS GENE

Background of the Invention

The present invention relates to a novel gene and polypeptides derived therefrom encoding a redox-sensitive protein that protects cells from apoptosis and promotes cell growth, as well as antibodies directed against the polypeptide. The invention also describes methods for using the novel gene, polypeptides, and antibodies in the detection of genetic deletions of the gene, subcellular localization of the polypeptide, isolation of discrete classes of RNA, inhibition of apoptosis, scavenging of oxygen radicals, reversion of tumor phenotype, and therapeutic applications by gene therapy.

Summary of the Related Art

Apoptosis, also referred to as programmed cell death, is a genetically programmed process for maintaining homeostasis under physiological conditions and for responding to various stimuli (Thompson (1995) Science 267, 1456-1462). This form of cell death is characterized by cell membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, and DNA fragmentation (Wyllie (1980) Int. Rev. Cytol. 68, 251-306). The process of apoptosis can be divided into three distinct phases: initiation, effector molecule stimulation and DNA degradation (Kroemer et al. (1995) FASEB J. 9, 1277-1287; Vaux and Strasser (1996) Proc. Natl. Acad. Sci. USA 93, 2239-2244). Apoptosis can be initiated in various cell types by a wide variety of physical, chemical, and biological stimuli (both internal and external), including diverse cancer therapeutic drugs, oxidative DNA damage reagents, and cytokines (Kroemer (1997) Nature Med. 3, 614-620, White (1996) Genes Dev. 10, 1-15; Sen and D'Incalci (1992) FEBS Lett. 307, 122-127; Dive and Hickman (1991) Br. J. Cancer 64, 192-196; Yuan et al. (1993) Cell 75, 641-652). These initiators trigger the effector molecules in cells leading to apoptotic signal transduction and amplification, which ultimately results in irreversible DNA degradation and cell death.

Many genes are involved in the apoptotic process. In general, the products of these genes are classified as either inducers or inhibitors of apoptosis. The balance between the activities of apoptosis inducers and inhibitors in a given cell determines whether that cell undergoes apoptosis. Among the growing list of apoptotic regulatory genes, the most well characterized are the p53 tumor suppressor gene, the Bcl-2 gene family (consisting of both inducers and inhibitors of apoptosis), the interleukin 1β converting enzyme (ICE) gene family, and FAS/Fas ligand (Kroemer (1997), White (1996); Yuan et al. (1993); Nagata and

Golstein (1995) Science 267, 1449-1456). During apoptosis, there are substantial interactions involving the products of apoptotic regulatory genes, including heterodimer formation among the gene products of the Bcl-2 gene family, and p53 activation of Bax expression (Oltvai et al. (1993) Cell 74, 609-619; Miyashita and Reed (1995) Cell 80, 293-299).

The inventor has recently found that 1,10 phenanthroline ("OP"), a metal chelating agent, can activate p53 activity and induce apoptosis in two murine tumor cell lines that harbor endogenous wild-type p53 (Sun et al. (1997) Oncogene 14, 385-393). OP is a typical metal chelating reagent in that it chelates Fe(II) and prevents Fe(II)-mediated hydroxyl radical formation through the Fenton reaction (Halliwell et al. (1989) in: Free Radicals in Biology and Medicine, 2nd ed., Clarendon Press, Oxford; Auld (1988) in Methods in Enzymology, Vol. 158 (J. F. Riordan and B. L. Valle, Eds.) PP. 110-114, Academic Press, New York). OP has been shown to prevent hydroxyl radical-induced DNA damage in a number of cellular systems (Sun, Y. Free Radic. Biol. Med. 8:583-599 (1990); Martins and Meneghini, Biochem J. 299:137-140 (1994); Morgan et al., Biochem. Pharmacol. 44:215-221 (1992)). Activation of p53 by OP was found to significantly contribute to, but was not required for subsequent apoptotic cell death (Sun et al., (1997) Oncogene 14: 385-393; Sun (1997) FEBS Lett. 408, 16-20). Thus, the critical genes and gene products responsible for OP-induced apoptosis remain to be characterized. A better understanding of the molecular mechanisms of apoptotic induction will allow improved design of therapeutic drugs that either induce (anti-cancer) or inhibit (anti-aging) apoptosis.

Summary of the Invention

The present invention provides novel genes and polypeptides derived therefrom encoding a redox-sensitive protein that protects cells from apoptosis, scavenges oxygen radicals, and can be used for the reversion of a tumor phenotype.

In one aspect, the present invention provides novel isolated and purified DNA sequences (referred to herein as "mouse SAG" and "human SAG") as shown in SEQ ID 1 and SEQ ID 3, and their gene products (referred to herein as "mouse SAG protein" and "human SAG protein") as shown in SEQ ID 2 and SEQ ID 4, that are induced during 1,10-phenanthroline ("OP")-induced apoptosis. In another embodiment, the present invention comprises a nucleotide sequence that hybridizes to the nucleotide sequence shown in SEQ ID 1 and SEQ ID 3 under high stringency hybridization conditions. In a preferred embodiment, the isolated and purified DNA sequence consists essentially of the DNA sequence of SEQ ID 1 or SEQ ID 3.

In another aspect, the invention provides novel recombinant DNA molecules, comprising SAG subcloned into an extra-chromosomal vector. In a further aspect, the present invention provides recombinant host cells that are stably transfected with a recombinant DNA molecule comprising SAG subcloned into an extra-chromosomal vector.

In a different aspect, the present invention provides a substantially purified recombinant protein comprising a polypeptide substantially similar to the SAG protein shown in SEQ ID 2 and SEQ ID 4. In a further aspect, the present invention provides a polyclonal antibody that selectively binds to proteins with an amino acid sequence substantially similar to the amino acid sequence shown in SEQ ID 2 and SEQ ID 4.

Additional aspects of the present invention provide a method of detecting the SAG protein in cells, comprising contacting cells with a polyclonal antibody that recognizes the SAG protein; a method of detecting cells containing SAG deletions, comprising isolating total genomic DNA from the cell and subjecting the genomic DNA to PCR amplification using primers derived from the DNA sequence of SEQ ID 1 and SEQ ID 3; and a method of detecting cells containing SAG deletions, comprising isolating total cell RNA and subjecting the RNA to reverse transcription-PCR amplification using primers derived from the DNA sequence of SEQ ID 1 and SEQ ID 3.

In another aspect, the present invention further provides methods of isolating RNA containing stretches of polyA, polyC, or polyU residues from cells, contacting the total cell RNA with the SAG protein, and incubating the RNA-SAG protein mixture with an antibody that recognizes the SAG protein.

In another aspect of the present invention, a method for isolating genes induced during cell apoptosis is provided, comprising treating cells with OP, subjecting the OP-induced RNA to the differential display procedure, and cloning the OP-induced genes.

A further aspect of the invention provides a method for protecting mammalian and/or non-mammalian cells from apoptosis induced by redox reagents, comprising introducing into mammalian and/or non-mammalian cells an expression vector comprising a DNA sequence substantially similar to the DNA sequence shown in SEQ ID 1 and SEQ ID 3, which is operatively linked to a DNA sequence that promotes the expression of the DNA sequence, wherein the isolated and purified DNA sequence of SEQ ID 1 and SEQ ID 3 will be expressed at high levels in the mammalian and/or non-mammalian cells.

An additional aspect of the present invention provides a method for treatment of mammalian and/or non-mammalian tumor cells, comprising introducing into mammalian and/or non-mammalian tumor cells an expression vector comprising a DNA sequence

substantially similar to the DNA sequence shown in SEQ ID 1 and SEQ ID 3, which is operatively linked to a DNA sequence that promotes the expression of the antisense strand of the DNA sequence, wherein the antisense strand of the DNA sequence of SEQ ID 1 and SEQ ID 3 will be expressed at high levels in the mammalian and/or non-mammalian cells.

Another aspect of the present invention provides a method for oxygen radical scavenging in an organism, comprising administering an oxygen radical-reducing amount of a pharmaceutical composition comprising SAG protein and a pharmaceutically acceptable carrier.

A further aspect of the present invention provides for gene therapy applications of SAG, including but not limited to methods of promoting the closure (i.e., healing) of a wound in a patient.

The foregoing is not intended and should not be construed as limiting the invention in any way. All patents and publications cited herein are incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1A. Predicted structural features of the deduced protein sequence of the mouse and human SAG cDNA.

Figure 1B. Description of human SAG protein mutants.

Figure 2. Bar graph depiction of soft agar colony growth of various SAG-transfected stable cell lines.

Figure 3. Graphical representation of tumor mass in SCID mice per days post implant with SAG transfectants.

Detailed Description of the Invention

The present invention provides novel genes and polypeptides derived therefrom encoding a redox-sensitive protein that protects cells from apoptosis, scavenges oxygen radicals, and can be used for the reversion of a tumor phenotype. The present invention also comprises genes and their gene products involved in OP-induced apoptosis. The isolation of such genes and their gene products permits a detailed analysis of the OP-induced apoptotic pathway, thus providing laboratory tools useful to identify the mechanisms of OP-induced apoptosis and enabling improved design of therapeutic drugs to regulate apoptosis.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual*

(Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), and *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

In one aspect, the present invention provides novel isolated and purified DNA sequences, hereinafter referred to as Sensitive to Apoptosis Genes ("SAG"), encoding SAG proteins. In one embodiment, the invention comprises DNA sequences substantially similar to those shown in SEQ ID 1 (mouse SAG) or SEQ ID 2 (human SAG), respectively. As defined herein, "substantially similar" includes identical sequences, as well as deletions, substitutions or additions to a DNA, RNA or protein sequence that maintain the function of the protein product and possess similar zinc-binding motifs. Preferably, the DNA sequences according to the invention consist essentially of the DNA sequence of SEQ ID 1 or SEQ ID 3, or are selected from the group consisting of SEQ ID 11, SEQ ID 13, SEQ ID 21, SEQ ID 23, SEQ ID 25, SEQ ID 27, SEQ ID 29, SEQ ID 31, SEQ ID 33, SEQ ID 35, SEQ ID 37, SEQ ID 39, SEQ ID 41, SEQ ID 43, SEQ ID 45, SEQ ID 47 and SEQ ID 49. These novel purified and isolated DNA sequences can be used to direct expression of the SAG protein and for mutational analysis of SAG protein function.

Mutated sequences according to the invention can be identified in a routine manner by those skilled in the art using the teachings provided herein, as described in Example 8, *infra*, and techniques well known in the art.

In another embodiment, the invention comprises a nucleotide sequence that hybridizes to SEQ ID 1 and/or SEQ ID 3 under high stringency hybridization conditions. As used herein, the term "high stringency hybridization conditions" refers to hybridization at 65°C in a low salt hybridization buffer to the probe of interest at 2 x 10^8 cpm/μg for between about 8 hours to 24 hours, followed by washing in 1% SDS, 20 mM phosphate buffer and 1 mM EDTA at 65°C, for between about 30 minutes to 4 hours. In a preferred embodiment, the low salt hybridization buffer comprises between, 0.5-10% SDS, and 0.05M and 0.5 M sodium phosphate. In a most preferred embodiment, the low salt hybridization buffer comprises, 7% SDS, and 0.125M sodium phosphate. These DNA sequences can be used to direct expression

of the SAG protein and for mutational analysis of SAG protein function, and are isolated via hybridization as described.

In another aspect, the invention provides novel recombinant DNA molecules, comprising SAG or a sequence substantially similar to it subcloned into an extra-chromosomal vector. This aspect of the invention allows for *in vitro* expression of the SAG gene, thus permitting an analysis of SAG gene regulation and SAG protein structure and function. As used herein, the term "extra-chromosomal vector" includes, but is not limited to, plasmids, bacteriophages, cosmids, retroviruses and artificial chromosomes. In a preferred embodiment, the extra-chromosomal vector comprises an expression vector that allows for SAG protein production when the recombinant DNA molecule is inserted into a host cell. Such vectors are well known in the art and include, but are not limited to, those with the T3 or T7 polymerase promoters, the SV40 promoter, the CMV promoter, or any promoter that either can direct gene expression, or that one wishes to test for the ability to direct gene expression. These recombinant vectors are produced via standard recombinant DNA protocols as described in the references cited above. This aspect of the invention allows for high level expression of the SAG protein.

In a further aspect, the present invention provides recombinant host cells that are stably transfected with a recombinant DNA molecule comprising SAG subcloned into an extra-chromosomal vector. The host cells of the present invention may be of any type, including, but not limited to, non-eukaryotic (e.g., bacterial), and eukaryotic such as fungal (e.g., yeast), plant, non-human animal, non-human mammalian (e.g., rabbit, porcine, mouse, horse) and human cells. Transfection of host cells with recombinant DNA molecules is well known in the art (Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, 1989) and, as used herein, includes, but is not limited to calcium phosphate transfection, dextran sulfate transfection, electroporation, lipofection and viral infection. This aspect of the invention allows for *in vitro* and *in vivo* expression of SAG and its gene product, thus enabling high-level expression of SAG protein, as described in Example 6, *infra*.

In another aspect, the present invention provides a substantially purified recombinant protein comprising a polypeptide substantially similar to the SAG polypeptides shown in SEQ ID 2 and SEQ ID 4. Furthermore, this aspect of the invention enables the use of SAG protein in several *in vitro* assays described below. As used herein, the term "substantially similar" includes deletions, substitutions and additions to the sequences of SEQ IDs 1-4 (as

appropriate) introduced by any *in vitro* means. As used herein, the term "substantially purified" means that the protein should be free from detectable contaminating protein, but the SAG protein may be co-purified with an interacting protein, or as an oligomer. Preferably, the protein sequences according to the invention comprise an amino acid sequence selected from the group consisting of SEQ ID 2, SEQ ID 4, SEQ ID 12, SEQ ID 14, SEQ ID 22, SEQ ID 24, SEQ ID 26, SEQ ID 28, SEQ ID 30, SEQ ID 32, SEQ ID 34, SEQ ID 36, SEQ ID 38, SEQ ID 40, SEQ ID 42, SEQ ID 44, SEQ ID 46, SEQ ID 48, and SEQ ID 50. In a most preferred embodiment, the protein sequences according to the invention comprise an amino acid sequence selected from the group consisting of SEQ ID 2 and SEQ ID 4. Mutated sequences according to the invention can be identified in a routine manner by those skilled in the art using the teachings provided herein and techniques well known in the art. This aspect of the invention provides a novel purified protein that can be used for *in vitro* assays, as described in Examples 12, *infra*, and as a component of a pharmaceutical composition for oxygen radical scavenging, described *infra*.

In a further aspect, the present invention provides antibodies and methods for detecting antibodies that selectively bind polypeptides with an amino acid sequence substantially similar to the amino acid sequence of SEQ ID 2 and SEQ ID 4. The antibody of the present invention can be a polyclonal or a monoclonal antibody, prepared by using all or part of the sequence of SEQ ID 2 or SEQ ID 4, or modified portions thereof, to elicit an immune response in a host animal according to standard techniques (Harlow and Lane (1988), eds. Antibody: A Laboratory Manual, Cold Spring Harbor Press). In a preferred embodiment, the entire polypeptide sequence of SEQ ID 2 or SEQ ID 4 is used to elicit the production of polyclonal antibodies in a host animal.

The method of detecting SAG antibodies comprises contacting cells with an antibody that recognizes SAG protein and incubating the cells in a manner that allows for detection of the SAG protein-antibody complex. Standard conditions for antibody detection of antigen can be used to accomplish this aspect of the invention (Harlow and Lane, 1988). This aspect of the invention permits the detection of SAG protein both *in vitro* and *in vivo*, as described in Examples 12 and 14, *infra*.

In a further aspect, the present invention provides a diagnostic assay for detecting cells containing SAG deletions, comprising isolating total genomic DNA from the cell and subjecting the genomic DNA to PCR amplification using primers derived from the DNA sequence of SEQ ID 1 SEQ ID 3, SEQ ID 11, SEQ ID 13, SEQ ID 21, SEQ ID 23, SEQ

ID 25, SEQ ID 27, SEQ ID 29, SEQ ID 31, SEQ ID 33, SEQ ID 35, SEQ ID 37, SEQ ID 39, SEQ ID 41, SEQ ID 43, SEQ ID 45, SEQ ID 47 and SEQ ID 49.

This aspect of the invention enables the detection of SAG deletions in any type of cell, and can be used in genetic testing or as a laboratory tool. The PCR primers can be chosen in any manner that allows the amplification of a SAG gene fragment large enough to be detected by gel electrophoresis. Detection can be by any method, including, but not limited to ethidium bromide staining of agarose or polyacrylamide gels, autoradiographic detection of radio-labeled SAG gene fragments, Southern blot hybridization, and DNA sequence analysis. In a preferred embodiment, detection is accomplished by polyacrylamide gel electrophoresis, followed by DNA sequence analysis to verify the identity of the deletions. PCR conditions are routinely determined based on the length and base-content of the primers selected according to techniques well known in the art (Sambrook et al., 1989).

An additional aspect of the present invention provides a diagnostic assay for detecting cells containing SAG deletions, comprising isolating total cell RNA and subjecting the RNA to reverse transcription-PCR amplification using primers derived from the DNA sequence of SEQ ID 1 SEQ ID 3, SEQ ID 11, SEQ ID 13, SEQ ID 21, SEQ ID 23, SEQ ID 25, SEQ ID 27, SEQ ID 29, SEQ ID 31, SEQ ID 33, SEQ ID 35, SEQ ID 37, SEQ ID 39, SEQ ID 41, SEQ ID 43, SEQ ID 45, SEQ ID 47 and SEQ ID 49. This aspect of the invention enables the detection of SAG deletions in any type of cell, and can be used in genetic testing or as a laboratory tool.

Reverse transcription is routinely accomplished via standards techniques (Ausubel et al., in Current Protocols in Molecular Biology, ed. John Wiley and Sons, Inc., 1994) and PCR is accomplished as described above.

In another aspect, the present invention provides methods of isolating RNA containing stretches of polyA (adenine), polyC (cytosine) or polyU (uridine) residues, comprising contacting an RNA sample with SAG protein, incubating the RNA-SAG protein mixture with an antibody that recognizes the SAG polypeptide, isolating the antibody-SAG protein-RNA complexes, and purifying the RNA away from the antibody-SAG protein complex. This aspect of the invention provides a novel *in vitro* method for isolating a discrete class of RNA. In a preferred embodiment, the RNA sample is contacted with SAG protein in the presence (for preferential isolation of polyA and polyC-containing RNAs), or absence (for preferential isolation of polyU-containing RNAs), of a reducing agent. Preferred reducing agents for use in this aspect of the invention include, but are not limited to DTT and

β-mercaptoethanol. The reducing agents are preferably used at a concentration of between about 50 mM and 1 M. Isolation of antibody-SAG protein-RNA complexes can be accomplished via standard techniques in the art, including, but not limited to the use of Protein-A conjugated to agarose or cellulose beads.

In a further aspect of the present invention, a method for isolating genes induced during cell apoptosis is provided, comprising treating one set of cells with OP and not treating a control set of cells, isolating RNA from each set of cells, subjecting the RNA from each set of cells to reverse transcription and PCR ("differential display"), identifying cDNAs that are expressed in the OP-treated set of cells and not in the control set of cells, and cloning the OP-induced cDNAs. This aspect of the invention provides a tool for isolating other genes that control the OP-induced apoptotic pathway and is useful both as a way to enable the design of therapeutic drugs that regulate apoptosis and as a laboratory tool to identify the mechanisms of OP-induced apoptosis. Details of the differential display technique, including selection of primers, are well known in the art (Liang and Pardee, Science 257:967-971, 1992). Reverse transcription and PCR conditions are routinely determined based on the length and base-content of the primers selected according to techniques well known in the art (Sambrook et al., 1989). In a preferred embodiment, OP is used at a concentration of between 50 μM and 300 μM. In a most preferred embodiment, OP is used at a concentration of between 100 μM and 150 μM.

A further aspect of the invention provides a method for protecting mammalian and/or non-mammalian cells from apoptosis induced by redox reagents, comprising introducing into mammalian and/or non-mammalian cells an expression vector comprising a DNA sequence substantially similar to the DNA sequence shown in SEQ ID 1 or SEQ ID 3, that is operatively linked to a DNA sequence that promotes the expression of the DNA sequence and incubating the cells under conditions wherein the DNA sequence of SEQ ID 1 or SEQ ID 3 will be expressed at high levels in the mammalian and/or non-mammalian cells. In a preferred embodiment, the DNA sequence consist essentially of SEQ ID 1 or SEQ ID 3. Suitable expression vectors are as described above. In a preferred embodiment, the coding region of the human SAG gene is subcloned into an expression vector under the transcriptional control of the cytomegalovirus (CMV) promoter to allow for constitutive SAG gene expression.

An additional aspect of the present invention provides a method for inhibiting the growth of mammalian and/or non-mammalian tumor cells, comprising introducing into

mammalian and/or non-mammalian tumor cells an expression vector comprising a DNA that is antisense to a sequence substantially similar to the DNA sequence shown in SEQ ID 1 or SEQ ID 3 that is operatively linked to a DNA sequence that promotes the expression of the antisense DNA sequence. The cells are then grown under conditions wherein the antisense DNA sequence of SEQ ID 1 or SEQ ID 3 will be expressed at high levels in the mammalian and/or non-mammalian cells. In a preferred embodiment, the DNA sequence consists essentially of SEQ ID 1, SEQ ID 3, SEQ ID 11, SEQ ID 13, SEQ ID 21, SEQ ID 23, SEQ ID 25, SEQ ID 27, SEQ ID 29, SEQ ID 31, SEQ ID 33, SEQ ID 35, SEQ ID 37, SEQ ID 39, SEQ ID 41, SEQ ID 43, SEQ ID 45, SEQ ID 47 and SEQ ID 49.

In a most preferred embodiment, the DNA sequence consists essentially of SEQ ID 1 or SEQ ID 3. In a further preferred embodiment, the expression vector comprises an adenoviral vector wherein SAG cDNA is operatively linked in an antisense orientation to a cytomegalovirus (CMV) promoter to allow for constitutive expression of the SAG antisense cDNA in a host cell. In a preferred embodiment, the SAG adenoviral expression vector is introduced into mammalian tumor cells by injection into a mammalian tumor cell mass.

An additional aspect of the present invention provides a method for oxygen radical scavenging in an organism, comprising introducing into mammalian and/or non-mammalian cells an expression vector comprising a DNA sequence substantially similar to the DNA sequence shown in SEQ ID 1 or SEQ ID 3 which is operatively linked to a DNA sequence that promotes the expression of the DNA sequence, and the cells are grown under conditions wherein the DNA sequence of SEQ ID 1 or SEQ ID 3 will be expressed at high levels in the mammalian and/or non-mammalian cells. In a preferred embodiment, the DNA sequence consists essentially of SEQ ID 1 or SEQ ID 3. In a preferred embodiment, the SAG cDNA is operatively linked to a cytomegalovirus (CMV) promoter, to allow for constitutive expression of the SAG cDNA in a host cell.

Another aspect of the present invention provides pharmaceutical compositions and methods for oxygen radical scavenging in an organism, comprising administering an oxygen-reducing amount of a pharmaceutical composition comprising the SAG protein of SEQ ID 2 or SEQ ID 4 and a pharmaceutically acceptable carrier.

Chimeric gene constructs of the present invention (e.g., expression vectors) containing SAG polynucleotide sequences may be used in gene therapy applications to achieve expression of SAG or anti-sense SAG polynucleotide sequences in selected target cells, including non-eukaryotic cells (i.e., plant) and eukaryotic cells. Gene therapy applications typically involve identifying target host cells or tissues in need of the therapy,

designing vector constructs capable of expressing a desired gene product in the identified cells, and delivering the constructs to the cells in a manner that results in efficient transduction of the target cells.

The cells or tissues targeted by gene therapy are typically those that are affected by the disease that the vector construct is designed to treat. For example, in the case of cancer, the targeted tissues are malignant tumors.

In one embodiment, the present invention provides a method of promoting the closure (i.e., healing) of a wound in a patient. This method involves transferring exogenous SAG to the region of the wound whereby a product of SAG is produced in the region of the wound to promote the closure (i.e., healing) of the wound.

The present inventive method promotes closure (i.e., healing) of both external (e.g., surface) and internal wounds. Wounds to which the present inventive method is useful in promoting closure (e.g., healing) include, but are not limited to, abrasions, avulsions, blowing wounds, burn wounds, contusions, gunshot wounds, incised wounds, open wounds, penetrating wounds, perforating wounds, puncture wounds, seton wounds, stab wounds, surgical wounds, subcutaneous wounds, tangential wounds, or traumatopneic wounds. Preferably, the present inventive methods are employed to close chronic open wounds, such as non-healing external ulcers and the like.

Exogenous SAG can be introduced into the region of the wound by any appropriate means, such as, for example, those means described herein. For example, where the wound is a surface wound, SAG can be supplied exogenously by topical administration of SAG protein to the region of the wound.

Preferably, exogenous SAG is provided to the wound by transferring a vector comprising an SAG expression cassette to cells associated with the wound. Upon expression of SAG within the cells in the region of the wound, a product of SAG is produced to promote wound closure (i.e., healing). Transferring a vector comprising an SAG expression cassette to cells associated with the wound is preferred as such procedure is minimally invasive, supplies SAG products locally within the region of the wound, and requires no reapplication of salves, solutions, or other extrinsic media. Furthermore, SAG activity remains expressed during wound closure and will inactivate following healing.

The vector comprising the SAG expression cassette can be transferred to the cells associated with the wound in any manner appropriate to transfer the specific vector type to the cells, such as those methods discussed herein.

As discussed above, the cells associated with the wound to which the vector is transferred are any cells sufficiently connected with the wound such that expression of SAG within those cells promotes wound closure (i.e., healing), such as cells within the wound or cells from other sources. In one embodiment, the cells are cells of the wound, and the present inventive method comprises transfer of the vector to the cells *in situ*.

In other embodiments, the cells are not the cells of the wound, but can be cells in an exogenous tissue, such as a graft, or can be cells *in vitro*. For example, to promote the healing of certain types of wounds, the cells associated with the wound can be cells within a graft, such as a skin graft. Transfer of the vector to the cells associated with the wound, thus involves transferring the vector to the cells within the graft *ex vivo*. For other wounds, the cells associated with the wound are cells *in vitro*, and the cells are transferred to the region of the wound following transfer to them of a vector containing the SAG expression cassette.

The present inventive method applies to any patient having a wound. For example, the patient can be any animal, such as a mammal. Preferably, the patient is human.

In another embodiment, the present invention provides a method of inhibiting or promoting plant cell growth. The method involves the use of chimeric gene constructs to achieve expression of SAG, in the case of promoting growth of plants, or anti-sense SAG, in the case of inhibiting plants (i.e., weeds), polynucleotide sequences in selected target plant cells.

The dosage regimen for *in vivo* oxygen radical scavenging by the administration of SAG protein is based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely using standard methods. In a preferred embodiment, the pharmaceutical composition comprises between 0.1 and 100 mg of SAG protein. In a most preferred embodiment, the pharmaceutical composition comprises between 1 and 10 mg of SAG protein.

The SAG protein may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The SAG protein may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

While the SAG protein can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other agents. When administered as a

combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

For administration, the SAG protein is ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The SAG protein may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the SAG protein may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

In a preferred embodiment of the present invention, the SAG protein pharmaceutical composition is administered intramuscularly (IM) or intravenously (IV). A suitable IM or IV dose of active ingredient of SAG protein is 5 mg/mL administered daily. For IM or IV administration, the active ingredient may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Examples

Example 1. Identification of an OP-inducible gene

The differential display (DD) technique was employed to isolate genes responsible for or associated with OP-induced apoptosis in two murine tumor lines. Since OP induced-apoptosis can be visually detected at 12 hours post exposure (Sun, (1997) FEBS Lett. 408:16-20), it was reasoned that gene(s) responsible for apoptosis induction should be up- or down-regulated prior to the appearance of apoptosis. Six hours of OP treatment was conducted, therefore, in one of these tumor lines followed by the DD analysis.

Mouse JB6 tumor line L-RT101 (an epidermal originated tumor cell line) was cultured in Minimal Essential Medium with Earle's salts (BRL) containing 5% fetal calf serum (Sigma). H-Tx cells, a spontaneously transformed mouse liver line, were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum and 1 mM sodium pyruvate. Human colon carcinoma line DLD-1 was grown in 10% DMEM.

L-RT101 cells were treated with 150 uM OP for 6 hours and subjected to differential display analysis using DMSO-treated cells as a control. Briefly, total RNA was isolated from both OP-treated and control cells using RNAzol solution (Tel-Test) according to the manufacturer's instructions, and subjected to reverse transcription (RT), performed as previously described (Sun et al. (1993) Mol. Carcinogenesis 8, 49-57), followed by the polymerase chain reaction (PCR). The primer used for reverse transcription (P1) consisted of the sequence 5 AAGCTTTTTTTTTTTTTR (SEQ ID 5), wherein R consists of either adenine, guanine or cytosine. P1 was used as the downstream primer in the subsequent PCR while the upstream primer consisted of the sequence AAGCTTNNNNNNN (SEQ ID 6), wherein N consists of adenine, cytosine, guanine, or thymine.

Primers P1 and P2 reproducibly detected differential expression between the control and OP-treated cells. The fragments reproducibly showing differential expression were PCR amplified using the same primers and used as probes for Northern analysis (Sun et al. (1992) Cancer Res. 52:1907-1915) of both L-RT101 and H-Tx cells treated with OP (Sun (1997) FEBS Letters 408:16-20). Those fragments that were induced by OP (as determined by Northern analysis) were then subcloned into TA cloning vectors (In Vitrogen) according to the manufacturer's instructions, and sequenced by DNA Sequenase Version 2.0, according to the manufacturer's instructions (Amersham). The resulting clones comprise OP-inducible cDNA fragments.

Example 2. cDNA library screening and 5'RACE

One of the OP-inducible clones was used as a probe to screen a mouse lung cDNA library to clone the full length mouse SAG cDNA. Briefly, 1 x 10^6 recombinant plaques were plated onto 1% NZY in 150 mm plates (a total of 20). The recombinant phage DNA was transferred to nitrocellulose membrane and hybridized with mouse SAG probe ($2X10^8$ cpm/μg) in a hybridization solution containing 5X SSC, 5X Denhardt solution, 50 mM sodium phosphate, and 100 μg/mL denatured DNA at 60°C for 16-18 hours. The filter was

then washed once for 5 min in a solution of 2XSSC/0.1% SDS, once for 5 min in 0.5XSSC/0.1% SDS, and twice 0.1XSSC/0.1% SDS for 15 min.

The longest clone isolated was a 1.0 kilobase ("kb") fragment consisting of a partial open reading frame and the entire 3'-end untranslated region. A mouse brain Marathon-Ready cDNA (ClonTech) was screened via PCR amplification using a primer derived from the 1 kb fragment and another primer derived from the vector sequence, according to the protocol supplied with the cDNA library. This yielded a further 100 bp fragment consisting of 5'-end untranslated sequence and some of the coding sequence. The derived cDNA clone consists of 1140 base pairs ("bp") (SEQ ID 1) that encode a novel deduced protein of 113 amino acids, containing 12 cysteine residues (SEQ ID 2). The open reading frame was preceded by 17 bp upstream sequence. The start codon was located in a context that conformed 100% to the Kozak consensus sequence (Kozak,M. (1991) J. Biol. Chem. 266, 19867-19870). An in-frame stop codon was identified 72 bp upstream of the start codon in the 5' untranslated region in one genomic clone (not shown). The 3'-end untranslated region consists of 792 bp sequence with two polyadenylation signals (AATAAA). These data indicate that a near full length cDNA was isolated.

The mouse cDNA was used as a probe to screen a human HeLa cell cDNA library (Strategene) as described above. One positive clone was isolated and purified through two more cycle of screening. In this manner, a 754 bp clone containing a polyadenylation signal at the 3' end was isolated (SEQ ID 3). The human cDNA also contains an open reading frame encoding a novel predicted 113 amino acid polypeptide containing 12 cysteine residues (SEQ ID 4). The sequence identity between the isolated mouse and human cDNAs is 82% in overall sequence and 94% in the coding region. At the protein level, they shared 96.5% identity, with all 12 cysteine residues being conserved. Computer analysis of protein databases using the GCG program (Genetics Computing Group, Madison, WI) revealed that the encoded proteins share 70% identity with hypothetical proteins from yeast (accession #Z74876) and C-elegans (accession #80449).

Motif searching of the deduced protein sequences using the GCG program did not reveal any known functional domains. However, they each contain two imperfect heme binding sites (CXXCH, at codons 47-51 and 50-54) (Matthews, Prog. Biophys. Mol. Biol. 45:1-56, 1985) and one imperfect C_3HC_4 zinc ring finger domain (Freemont et al., Cell 64:483-484, 1991) at the C-terminal of the molecule (Fig. 1A) among other consensus motifs. The second potential heme binding domain (Fig. 1A) contains a substitution of arginine to

histidine (amino acid 54). Since these two amino acids are structurally similar, this may constitute an authentic heme binding site. The zinc ring finger domain mismatch involves substitution of cysteine by histidine at amino acid 85. The ring finger domain in this protein is a $C_3H_2C_3$ structure, rather than the consensus C_3HC_4 structure. Since cysteine and histidine residues are interchangeable in zinc binding (Berg and Shi, Science 271:1081, 1996; Inouye et al., Science 278:103-106, 1997), the $C_3H_2C_3$ domain in these proteins may comprise authentic zinc-binding sites. Significantly, these heme and zinc ring finger domains are 100% conserved among *C. elegans*, mouse and human. In yeast, only the last cysteine residue in $C_3H_2C_3$ motif was not conserved. This evolutionary conservation of the heme and zinc-binding domains suggest their functional importance.

Other motifs identified in the deduced sequence of the SAG protein, when allowing for a single mismatch, include an aminoacyl-transfer RNA synthetase class II motif (codons 54-63), a Kazal serine protease inhibitor family motif (codons 85-107), a Ly-6/U-par domain (codons 65-107), a prokaryotic membrane lipoprotein lipid attachment site (codons 16-27), and somatotropin, prolactin and related hormone motifs (codons 49-66).

These experiments thus resulted in the cloning of novel mouse and human genes that encode nearly identical, evolutionarily conserved protein that contain distinct heme and zinc binding motifs.

Example 3. SAG is inducible by OP in both mouse and human tumor cells

To confirm that the cloned cDNAs are subject to OP induction, a Northern analysis was performed with RNAs isolated from mouse tumor lines L-RT101 and H-Tx, and human colon carcinoma line DLD-1. Subconfluent cells were treated with 150 μM OP for various times up to 24 hours and subjected to total RNA isolation. Fifteen μg of total RNA was subjected to Northern analysis using mouse SAG or human SAG cDNA as probes.

Both cloned mouse and human cDNAs detected an OP inducible transcript with a size of 1.2 kb and 0.9 kb, respectively. Since these genes were induced in the OP-induced apoptosis pathway, the genes were named Sensitive to Apoptosis Genes (hereinafter referred to as "SAG"), which encode SAG proteins.

Example 4. Tissue distribution and embryonic expression of SAG

SAG expression was next examined in multiple human tissues. The assays were performed as detailed previously (Sun et al. (1993) Mol. Carcinogenesis 8, 49-57; Sun et al., Proc. Natl. Acad. Sci. USA 90:2827-2831, 1993). Briefly, total RNA was isolated from

multiple human tissues (ClonTech) and then subjected to Northern blot analysis using the mouse or human SAG cDNA as probes. SAG RNA was detected in all tissue examined including heart, brain, pancreas, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. A very high expression level was detected in heart, skeleton muscle and testis, which consume high levels of oxygen. Its tissue distribution and high level expression in oxygen-consuming tissues, and its induction by a redox sensitive compound (OP), implies that SAG encodes a redox sensitive protein.

Since SAG protein is evolutionarily conserved, the possible developmental role of SAG was tested by measuring SAG expression in mouse embryonic tissue (provided by Dr. Tom Glaser, University of Michigan), using reverse transcription of total RNA followed by PCR with the following primers: SAGTA.01 5′-CGGGATCCCCATGGCCGACGTGAGG-3′ (SEQ ID 7) and SAGT.02 5′-CGGGATCCTCATTTGCCGATTCTTTG-3′ (SEQ ID 8), which flank the entire SAG coding region. The PCR reaction mixture for 11 samples contained 55 µL of 10X buffer, 22 µL of 1.25 mM dNTP, 1.1 µL of SAGTA.01 and SAGT.02, respectively, 5.5 µL of Taq DNA polymerase, 5.5 µL of ^{32}P-dCTP and sterile water up to 495 µl. Into each tube which contains 5 µL of first strand cDNA reverse-transcribed for total RNA isolated from mouse embryonic tissues (Sun et al. (1997), Mol. Carcinogenesis 8:49-57), 45 µL of reaction mixture was added and PCR was performed for 25 cycles (95°C for 45 sec, 60°C for 1 min and 72°C for 2 min). A 5 µL aliquot of the PCR product was denatured and separated on a sequencing gel, which was dried and exposed to X-ray film.

SAG RNA was expressed in 9.5 day old to 19.5 day old whole mouse embryos, with a higher level of expression detected between days 9.5 and 11.5. These results suggest that SAG plays a role in embryonic development.

Example 5. Cellular localization by immunofluorescence

NIH3T3 cells (ATCC CRL 1658) were plated on coverslips in 24-well culture dishes and transfected by the calcium phosphate method according to standard techniques (Sambrook et al, 1989) with the following constructs: pcDNA3.1 (Invitrogen vector pcDNA 3 with a myc-his-tag); pcDNA3.1-SAG (human SAG cDNA subcloned into the BamHI site of pcDNA3.1, downstream from the CMV promoter and upstream and in-frame with the myc-his-tag, such that upon expression, the resulting fusion protein consists of the SAG

protein followed by the myc-his tag at the carboxy-end of SAG); or pcDNA3.1-LacZ (Invitrogen). Two days post-transfection, cells were washed once with cold PBS and then fixed with 3% formaldehyde in PBS for 10 minutes followed by 5 minutes in 1:1 methanol:acetone. The fixed cells were washed 4 times in PBS and incubated with antibody directed against the Myc-tag (Invitrogen 1:200 dilution) in PBS containing 1% BSA, 0.1% saponin, 2 μg/mL DAPI for 1 hour in the dark with shaking. Cells were then washed 4 times with 0.1% saponin in PBS and incubated with FITC-conjugated goat anti-mouse antibody (Jackson Laboratory, 1:100 dilution) for 1 hour in the same conditions as the first antibody. After incubation cells were washed 4 times with 0.1% saponin in PBS and twice with PBS. The coverslips were then mounted to glass slides with non-fade mounting medium and analyzed using a Leita Dialux 20 microscope.

SAG fusion protein was detected in both the cytoplasm and nucleus, while the β-galactosidase control was expressed predominately in the cytoplasm. No immunofluorescence staining was detected with the vector-only control. The cytoplasmic/ nuclear localization of SAG was confirmed also in a SAG stable transfectant using both SAG and myc-tag antibodies. These data demonstrate that exogenously expressed SAG fusion proteins can be detected within transfected cells by using antibodies directed against an epitope fused to SAG protein.

Example 6. Expression and purification of SAG protein in bacteria

The entire open reading frame of the human SAG cDNA was PCR amplified as described above and subcloned into the pET11 expression vector (Novogen) under control of the T7 promoter, yielding construct pET11a-hSAG. The sequence and orientation of the SAG DNA insert were confirmed by DNA sequencing. pET11a-hSAG was used to transform *E. coli* strain BL21 (Novagen, Inc.). Transformed cells were grown in LB media containing ampicillin (50 μg/mL). SAG expression was induced by 0.5 mM IPTG and SAG protein was found in inclusion bodies, which were subsequently isolated as follows.

Following IPTG induction, four liters of cells were grown for 4.5 hours at 37°C at a shaker setting of 150 rpm. Cell pellets were obtained by centrifugation at 5000 rpm for 10 minutes, and were resuspended in 100 mL TN buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl) containing 100 μM PMSF. The resuspended cell pellet was subsequently sonicated (15 sec/round for 5 rounds at a setting of 15 on Model 50 sonic dismembrator, Fisher Scientific) and subjected to pressure of 2500 pounds/square inch on a French cell press,

followed by addition of 1 mM MgCl2 and 10 mg of DNase I. The cell lysate was placed on ice for 30-60 minutes and then centrifuged at 18,000 rpm and the supernatant was disposed.

The pellet was seen to have 2 layers. The white layer on the top was carefully blown loose with TN buffer and removed. The remaining dark brown layer on the bottom was resuspended thoroughly in 15 mL of urea buffer (7 M urea, 20 mM Tris-HCl, pH 7.5, 200 mM NaCl) and allowed to sit overnight at room temperature. The resuspended cell pellet was vigorously homogneized with a serological pipette and then centrifuged at 40,000 rpm for 40 minutes using an SW50 ultracentrifuge rotor. The supernatant was collected and concentrated using a Centricon-10 concentrator to a volume of 5 mL and loaded onto a Sephacryl-100 column (100 cm long with a diameter of 2.5 cm) that had been equilibrated with urea buffer. The column was run at a rate of 0.25 mL/min and fractions were collected. The early fractions containing a brownish color consisted of mostly the large molecular weight protein, as expected. They also contained a protein with the same size of SAG protein (approximately 13 kDa). Since SAG protein contains 12 cysteine residues, it follows that SAG protein may form oligomers when expressed in bacteria and thus may elute as a SAG protein oligomer. Since SAG is a redox-sensitive protein, the DTT present in SDS sample buffer reduces SAG protein oligomers to monomer, leading to the detection of a fast migrating band. When early fractions were run in SDS-PAGE without DTT, the 13 kDa SAG protein band disappeared, and a 260 kDa band was detected, representing a SAG protein 20-mer. This unique feature helped us to purify SAG protein. Early fractions were pooled and loaded on the same Sephacryl-100 column pre-equilibrated with 7M urea and 5mM DTT.

SAG protein oligomer was reduced to monomer by using DTT in the loading buffer and was eluted in the later fractions, thus separating it from high molecular weight contaminant proteins (eluted earlier). The brownish fractions were pooled and concentrated using a Centricon-10 to a volume of 5 mL. DTT was added to a concentration of 5 mM. The combined fractions were loaded onto an S-100 column (100 cm long with a diameter of 2.5 cm), that had been equilibrated with urea buffer plus 5 mM DTT. The column was run at a rate of 0.25 mL/min and fractions were collected. The fractions containing SAG protein are brownish in color, highly suggesting that SAG is a heme-containing protein. The SAG protein containing fractions and their sensitivity to DTT were confirmed by Western blot using SAG antibody. The brownish fractions were pooled and concentrated using a Centricon-10 concentrator to a volume of 2 mL. The resulting sample was dialyzed against 4 liters of dialysis buffer (150 mM KCl, 20 mM Tris-HCl, pH 7.5) at 4°C overnight to

remove urea and DTT to yield refolded SAG protein. The dialyzed sample was loaded onto an S-100 column (100 cm long with a diameter of 2.5 cm), that had been equilibrated with dialysis buffer. The brownish fractions were pooled and concentrated using a Centricon-10 to a volume of 1 mL. The resulting sample was stored at 4°C. The protein concentration was determined by a BioRad protein assay. The purity of the samples was demonstrated in 10-20% SDS-PAGE. These data demonstrate the purification of recombinant SAG protein.

Example 7. Redox Sensitivity of SAG Protein

To confirm that purified recombinant SAG protein possesses the same redox sensitivity as it shows during protein purification, the sensitivity of refolded SAG to redox reagents was examined next. SAG protein (1 μg) was exposed to various concentrations of DTT (1 M, 300 mM, 100 mM, or 30 mM) or H_2O_2 (15 mM, 50 mM, 150 mM or 450 mM) for 10 min before being separated by polyacrylamide gel electrophoresis (PAGE), followed by Western blot analysis. Alternatively, 10 μg of SAG protein was incubated with 50 mM H_2O_2 for 10, 30, 60 or 120 minutes followed by PAGE separation and Coomassie Blue staining.

Dimers of SAG protein are rather resistant to reducing reagent DTT since no significant dimer was reduced to monomer after DTT treatment. However, as little as 15 mM H_2O_2 induces oligomerization of SAG protein, possibly through the formation of intermolecular disulfide bonds. The oligomerization is incubation-time dependent, as higher order SAG protein oligomers were detected upon increased incubation time. Interestingly, a band migrating faster than the monomer form is observed upon H_2O_2 treatment, and the monomer form of SAG protein becomes a doublet, possibly due to the formation of intramolecular disulfide bonds.

In order to determine whether H_2O_2-induced SAG protein oligomerization can be reversed by DTT treatment, 1 μg of purified SAG protein was incubated with 50 mM H_2O_2 for 10 minutes, followed by a 10 minute incubation with either H_2O_2, 50 mM DTT, 100 mM, 500 mM, or 1 M DTT. The samples were separated via PAGE followed by Western analysis. The results demonstrated that H_2O_2-induced SAG protein oligomerization can be reversed by subsequent incubation with DTT in a dose dependent manner, indicating that SAG protein oligomerization is subject to redox regulation.

To confirm that SAG protein oligomerization and doublet formation is due to inter- and intra-molecular disulfide bond formation, respectively, SAG protein was treated, prior to H_2O_2 exposure, with 50 mM N-ethylmaleimide (NEM), an alkylating reagent that will alkylate the free SH-groups in SAG protein. Purified SAG protein (1 μg) was pre-incubated with 50 mM NEM or DMSO, or buffer only, for 10 minutes prior to H_2O_2 treatment. The samples were separated via PAGE, followed by Western blot analysis. Pre-incubation of SAG protein with DMSO did not affect H_2O_2-induced oligomerization and doublet formation, whereas NEM pre-treatment abolished H_2O_2 activity. Neither inter- (oligomerization) nor intra- (doublet monomer) disulfide bonds were formed, demonstrating that alkylation of the free SAG protein SH groups abolishes H_2O_2 sensitivity. These data demonstrate that SAG protein is redox sensitive. It is subjected to both intra- and inter-molecular disulfide bond formation upon exposure to H_2O_2, as evidenced by both doublet and oligomer formation. These H_2O_2-induced changes can be reversed by subsequent treatment with reducing reagents, including DTT, or can be prevented by NEM pretreatment. It has also been observed that zinc can promote H_2O_2-induced oligomerization, although zinc itself did not induce oligomerization.

Example 8: Production of SAG mutants

In order to understand the role of each particular cysteine residues in heme binding and SAG oligomerization, a series of single and double SAG mutants were made in heme binding sites as well as the zinc ring finger motif (see Figure 1B). To generate single point mutations in SAG cDNA, 15 pairs of sense and antisense primers were designed, which are partially complimentary and contain a desired point mutation. The wildtype SAG cDNA cloned into the pET11a vector at the Nhe I/Bam HI sites was used as the template for PCR amplification. Two separate PCR reactions were conducted using a) primer SAG P.01 (5′-TATGGCTAGC ATGGCCGACGTGGAGG-3) (**SEQ ID 9**) and each of antisense primers and b) each of sense primers and SAG T.02 (**SEQ ID 8**), respectively. The resultant PCR products that overlap with each other and contain a desired point mutation were mixed and served as templates for a third PCR. The primers used were SAG P.01 and SAG T.02, which flank the entire encoding region of SAG cDNA. The PCR was performed as previously described (Sun et al. (1992) BioTechniques 12:639-640). The PCR products were digested with restriction enzymes Nhe I and Bam HI and subcloned into the pET11a vector, which was digested with the same restriction enzymes. To generate SAG double mutants

(MM10, MM13, MM14, see Figure 1B), a QuickChange site-directed mutagenesis kit was purchased from Strategene (La Jolla, CA) and used as instructed. All SAG mutants generated were verified by DNA sequencing (SEQ ID 21, SEQ ID 23, SEQ ID 25, SEQ ID 27, SEQ ID 29, SEQ ID 31, SEQ ID 33, SEQ ID 35, SEQ ID 37, SEQ ID 39, SEQ ID 41, SEQ ID 43, SEQ ID 45, SEQ ID 47 and SEQ ID 49). The predicted mutant SAG proteins encoded by these mutant SAGs are shown in SEQ ID 22, SEQ ID 24, SEQ ID 26, SEQ ID 28, SEQ ID 30, SEQ ID 32, SEQ ID 34, SEQ ID 36, SEQ ID 38, SEQ ID 40, SEQ ID 42, SEQ ID 44, SEQ ID 46, SEQ ID 48, and SEQ ID 50.

Individual SAG mutant-expressing vectors were used to transform *E.coli* strain BL21 (Novagen, Inc.). Mutant SAG protein was expressed and purified as detailed in Example 6. The fractions after a Sephacryl-100 column were collected and analyzed on 8-25% Phast gels followed by Coomassie blue protein staining. The pure fraction containing mutant SAG protein was dialyzed in 4 liters of 20 mM Tris-HCl, pH 7.5 and used for SAG protein oligomerization studies.

Purified wildtype SAG protein is a heme-containing brownish protein (See Example 9). Some of the purified SAG protein mutants were found to have either lost the brownish color (MM3 and MM13) or had decreased brownish color (MM1) compared to wildtype SAG protein. This color change indicates the loss or decrease of heme binding (Table 1).

TABLE 1. SUMMARY OF SAG MUTANTS

NAME	*MUTATION SITE(S)*	*HEME BINDING*	*OLIGOMERIZATION*
WT	None	+++	Yes
MM1	C_A/heme	++	Yes
MM2	C_B/heme	+++	Yes
MM3	C_{A+B}/heme	+/-	Yes
MM4	C_1/Zn-ring finger 1	+++	Yes
MM5	C_3/Zn-ring finger 1	+++	Yes
MM6	H_4/Zn-ring finger 1	+++	Yes
MM7	H_5/Zn-ring finger 2	+++	Yes
MM8	C_6/Zn-ring finger 2	+++	Yes
MM9	C_7/Zn-ring finger 2	+++	Yes
MM10	H_{4+5}/Zn-ring fingers 1&2	+++	Yes
MM11	C_2/Zn-ring finger 1	+++	Yes
MM12	C_c/protease inhibitor	+++	Yes
MM13	C_{1+2}/Zn-ring finger 1	+/-	Yes
MM14	C_{7+8}/Zn-ring finger 2	+++	No
MM15	GADPH binding site	+++	Yes

To examine mutant SAG protein oligomerization, each mutant SAG protein as well as wildtype SAG was treated with 50 mM H_2O_2 for 10 min. All of the SAG mutants, except MM14, can be oligomerized upon exposure to H_2O_2. The mutant 14, which is a double mutants in positions of C7 and C8 in the zinc ring finger domain, becomes insensitive to oligomerization (Table 1), indicating that these two positions are important for intermolecular disulfide bond formation.

Example 9. Heme measurement of SAG protein

Heme content in SAG protein was measured as previously described (Rieske (1967) Methods in Enzymol. 76, 488-493). Briefly, 1 mg of purified SAG protein, along with cytochrome C, catalase, and BSA as controls, was extracted with cold acetone (0.5 mLs)

After centrifugation the pellet was extracted sequentially with 0.5 mL of chloroform:methanol (2:1); 0.5 mL of cold acetone, and finally 0.5 mL of cold acetone containing 5 μL of 2.4 N HCl. The acetone extracts were dried under speed-vac and dissolved in 0.5 mL of pyridine. After addition of 0.5 mL of 0.2 N NaOH, the solution was centrifuged briefly and clear supernatant was recovered. One drop of diluted potassium ferricyanide (0.05 M) was added to the supernatant and the absorbance was read at 556 nm in 1.0 mL quartz cuvettes using water as a blank. The solution was then reduced by adding 10 uL of 2 M DTT and absorbance was read at 556 nm, 587 nm and 550 nm, respectively.

Heme absorbance at 556, 587, and 550 nm was observed in SAG protein, as well as in cytochrome C and catalase, but not in BSA. This result demonstrated that SAG protein contains heme, but did not reveal the molar ratio between SAG protein and heme molecule.

Example 10. SAG protein antibody production

Two polyclonal antibodies against SAG protein were generated using standard methods [by Zymed Laboratories, Inc. (San Francisco) under a service agreement with Warner-Lambert]. Briefly, the peptide antibody was generated as following. A 16-amino-acid peptide (SAG-Pep1: QNNRCPLCQQDWVVQR) (SEQ ID 10) located in the C terminus of SAG protein (codons 95-110) was synthesized and purified via standard techniques. The purified peptide was conjugated to keyhole limpet hemocyanin (KLH) via cysteine residues. The conjugated peptide (0.5 mg) was emulsified with equal volume of Complete Freund Adjuvant (CFA) and subcutaneously injected into rabbit, followed by 4 boosts with 0.5 mg each in Incomplete Freund Adjuvant (IFA) at 3 week intervals. Rabbits were bled 10 days after the final boost and antiserum was collected. The same protocol was used for protein antibody production using purified human SAG protein as the antigen, prepared as described above.

Example 11. Analysis of SAG protein transcriptional regulatory activity

SAG protein belongs to the zinc ring finger protein families by virtue of its $C_3H_2C_3$ motif (Saurin et al. (1996) TIBS 21, 208-214). Some zinc ring finger proteins have been shown to bind to DNA and function as transcriptional repressors (for example, RING1) (Satijn et al. (1997) Mol. Cell. Biol. 17, 4105-4113), whereas others function as transcriptional activators (Chapman and Verma (1996) Nature 382, 678-679; Monteiro et al. (1996) Proc. Natl. Acad. Sci. USA 93, 13595-13599). To examine the transcriptional regulatory activity of SAG protein, the cDNA encoding the entire open reading frame of

human SAG was PCR amplified and fused both in frame and as an antisense fusion, downstream of the Gal-4 DNA binding domain (encoding amino acids 1-147) in the pG4 vector (Sadowski et al., Nature 335:563-564, 1988). The resulting construct was sequenced to confirm in frame fusion and freedom from PCR-generated mutation. The construct was co-transfected along with a chloramphenicol acetyltransferase (CAT)-reporter-expressing vector (Sadowski et al., Nature 335:563-564, 1988) as well as a β-galactosidase reporter whose expression is driven by a CMV promoter for normalization of transfection efficiency into human kidney 293 cells (ATCC accession number CRL1573) by the calcium phosphate method. CAT activity was measured 36 hours post-transfection using a CAT assay kit (Quan-T-CAT; Amersham) according to the manufacturer's instructions. PG4-VP16, a known transcription factor (Triezenberg et al., Genes and Develop. 2:718-729, 1988), fused downstream of the Gal4 DNA binding domain was used as a positive control. Activation was calculated by arbitrarily choosing CAT activity from the vector control as 1 and comparing the other constructs to it. Three independent transfections and assays were performed.

SAG protein showed no transactivation activity. The positive control, VP16 showed 300-fold activation of CAT activity. To test for transrepression activity, SAG constructs (both sense and antisense) were co-transfected with pG4-VP16. Again, neither orientation of SAG induced significant expression of VP16-induced transactivation. These results demonstrated that SAG protein lacks transcriptional regulatory activity when fused downstream Gal-4 DNA binding domain.

Example 12. SAG is an RNA binding protein

The zinc-ring finger domain of the MDM2 protein has been shown to bind to RNA (Elenbaas et al. (1996) Mol. Med. 2, 439-445). Since SAG protein showed no transcriptional regulatory activity, it was tested whether SAG protein could bind to RNA or DNA. Binding of purified SAG protein to different nucleic acid cellulose conjugates was performed as described (Elenbaas et al. (1996)). Briefly, 0.5 μg of SAG protein was incubated in 300 μL RNA binding buffer for 1 hour at 4°C with double-stranded calf thymus DNA, denatured calf thymus DNA (ssDNA), or one of 4 RNA homopolymer columns (Sigma) conjugated to agarose or cellulose beads (Sigma), and used according to the manufacturer's instructions. RNA binding buffer consisted of 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% nonidet P-40, 50 μM $ZnCl_2$, 2% glycerol, and 1 mM DTT. The columns were washed with 3 mL RNA binding buffer to remove non-specifically bound protein from the beads, which

were then boiled in SDS sample buffer. The protein so eluted from the beads was separated by SDS-PAGE, transferred to nitrocellulose for Western blot analysis using the polyclonal antibody directed against SAG protein described previously detected by ECL chemiluminescence (Amersham) according to the manufacturer's instructions.

Purified SAG bound to polyU, polyA, and polyC RNA, respectively. No binding was seen with polyG RNA or ssDNA. A band showing dsDNA binding did not agree with SAG molecular weigh. Oligomeric SAG protein bound to polyU RNA, whereas the monomeric form of SAG binds to polyA and polyC RNA. Purified SAG protein was run as a marker. These results suggest that SAG is an RNA binding protein and that binding specificity is determined by the oligomeric form of SAG protein.

Example 13. Identification of two deletion mutants of SAG in cancer cell lines

Total RNA was isolated from DLD-1 colon carcinoma cells (ATCC accession number CCL221) and subjected to RT-PCR using primers SAG TA.01 and SAG T.02. The resulting PCR fragments were subcloned into the TA cloning vector (Invitrogen). During sequence verification of the resulting clones, it was found that several clones contained either a 7 bp or a 48 bp deletion at nucleotide 170 or 177, respectively, assigning the first A at the start codon as nucleotide #1. Both SAG deletions encode the potential heme-binding sites. The 7 base pair deletion (SAG mutant 1) (SEQ ID 11) is a frame shift deletion that abolishes the downstream encoded zinc-ring finger motif in the resulting protein (SEQ ID 12), whereas the 48 base pair deletion (SAG mutant 2) (SEQ ID 13) is an in-frame deletion that eliminates 16 amino acids in the encoded protein (SEQ ID 14), but retains the zinc-ring finger motif.

Total RNA was isolated from a total of 20 human tumor lines and transformed lines originating from lung, brain, kidney, prostate, testis, nasopharynx, bone, cervix and foreskin and subjected to RT-PCR analysis as described previously (Sun et al. (1993) Mol. Carcinogenesis 8, 49-57). Genomic DNA was also isolated from these cell lines and subjected to PCR amplification as described (Sun et al. (1992) BioTechniques 12:639-640). The primers used for PCR were hSAG.M1, 5′ GCCATCTGCAGGGTCCAG-3′ (SEQ ID 15), starting at nt 151 of hSAG cDNA, and SAGT.02-1 5′–GGATCCTCATTTGCCGATTCTTTGGAC-3′ (SEQ ID 16), including stop codon (underlined). The resulting fragment is 200 bp for wildtype SAG. The PCR was conducted in the presence of ^{35}S-dATP (Amersham) and PCR products were resolved in 6% denaturing sequencing gels, as described previously (Sun et al. (1995) Cancer Epidemiology, Biomarkers & Prevention, 4, 261-267). The bands corresponding to wildtype as well as the

two deletion mutants were cut out from the gel, PCR amplified using the same set of primers, and sequenced to verify the DNA sequence of the resulting PCR fragments.

Both the 7 base pair and the 48 base pair deletions were detected in RNA from only the CATES-1B cell line, a testicular carcinoma line obtained from ATCC (accession number HTB104). This tumor line also contains the wildtype SAG DNA sequence. The identity of these three bands was confirmed by DNA sequencing after PCR amplification and TA cloning. HONE-1, a nasopharyngeal carcinoma line which only contains wildtype SAG was included for comparison.

It was next examined whether these SAG deletions were detectable at the DNA level. Genomic DNA was isolated from CATES-1B cells and subjected to PCR analysis, as described previously (Sun et al. (1992) BioTechniques 12:639-640). The primers used were hSAG.M1 and SAG T.02 (see above for sequences). Genomic DNA from CATES-1B cells possesses only wildtype SAG and no SAG deletion mutants were detected. These results indicate that the SAG deletion mutations occur very rarely in human cancer lines. Detection of the mutations in SAG RNA, but not genomic DNA, may reflect an RNA editing modification of SAG messenger RNA.

Example 14. Production of stable SAG transfected mammalian cells

The potential biological function of human SAG protein was examined next by its overexpression in cells. DLD-1 cells were transfected with the following plasmids: the neo control pcDNA-3 (Invitrogen) (identical to pcDNA3.1 described above, except that it lacks the myc-his tag), pcDNA-SAG, pcDNA-SAG-mutant-1, and pcDNA-SAG-mutant-2 (pcDNA3 with SAG, SAG 1 or SAG 2 subcloned into the BamHI site, respectively, using methods well known in the art). The SAG mutant constructs were generated by RT-PCR as follows. Total RNA was isolated from DLD-1 cells, and subjected to reverse transcription, followed by PCR amplification. The primers used were SAG.TA01 (**SEQ ID 7**) and SAGT.02 (**SEQ ID 8**), which flank the entire coding region of SAG gene. The PCR products were digested with restriction enzyme Bam HI, and subcloned into pcDNA3 (In Vitrogen, San Diego), a mammalian expression vector under the transcriptional control of the CMV promoter, which drives gene expression constitutively. The resultant clones were sequenced to confirm both sense and antisense orientation and freedom of PCR-generated mutations. DNA sequencing revealed wildtype SAG clone as well as two deletion mutants: SAG-mutant-1 (7 bp deletion, SEQ ID 11) and SAG-mutant-2 (48 bp deletion, SEQ ID 13) in DLD-1 tumor cells.

DLD-1 cells were transfected by lipofectamine (BRL) with plasmids expressing wildtype (both sense and antisense orientation), SAG mutant-1, and SAG mutant-2, along with the neo control vector. Neomycin resistant colonies were identified by G418 selection (600 μg/mL) for 18 days. Stable clones were ring-isolated by well known methods (Sun et al. (1993) Proc. Natl. Acad. Sci. USA. 90: 2827-2831) and SAG expression was monitored by Northern analysis. Selected clones were examined for SAG protein expression by immunoprecipitation, as described below.

Total RNA was isolated from the cloned cell lines and subjected to Northern analysis. Cell lines transfected with the following constructs were analyzed: vector controls D1-3 and D1-6; SAG-wildtype D12-1 and D12-8; SAG-mutant-1 D3-3 and D3-4; and SAG-mutant-2 D4-2 and D4-5.

Northern blot analysis of RNA from selected stable SAG-expressing clones probed with the human SAG cDNA demonstrated that all SAG transfectants express SAG mRNA, while very low levels of endogenous SAG message were detected in the neo control cells.

The vector control lines and SAG wildtype and SAG deletion mutant transfectants were subsequently subjected to immunoprecipitation using standard techniques (Sun et al. (1993) Proc. Natl. Acad. Sci. USA. 90: 2827-2831, Sun et al. (1993) Mol. Carcinogenesis 8, 49-57). Subconfluent SAG transfectants were subjected to methionine starvation for 1 hour and then metabolically labeled with ^{35}S-translabel (0.2 mCi/mL) for 3 hours. Cells were then lysed on ice for 30 minutes in a lysis buffer comprising 2% Nonidet P40, 0.2% SDS, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μl/mL leupeptin, and centrifuged at 12,000 x g. The TCA precipitable radioactivity in the supernatant (1 x 10^8 cpm) was immunoprecipitated using rabbit anti-human SAG antibody (generated as described above). The immunoprecipitates were collected, washed, and analyzed on a 10-20% SDS-polyacrylamide gel, followed by autoradiography. High SAG protein expression was detected only in the wildtype transfectants. The antibody used did not recognize the two SAG protein mutants. These data demonstrate the production of stably transfected cells expressing either wildtype or mutant SAG protein

Example 15. Morphological appearance of SAG transfectants after exposure to redox reagents

Two neo controls (D1-3 and D1-6) and two SAG-producing lines (D12-1 and D12-8) were chosen to examine their sensitivity to redox compounds by morphological observation. After exposure to 150 μM OP, 200 μM H_2O_2, or 125 μM zinc for 24 hours, the neo-control cells were shrunken and detached, a sign of apoptosis, while SAG-expressing cells appeared morphologically normal. These results indicate that SAG production protects cells from apoptosis induced by redox compounds. Expression of SAG, however, did not offer the protection against copper. No difference in morphological signs of apoptosis was observed with $CuSO_4$ treatment (up to 750 μM) between the vector controls and SAG transfectants. Higher doses induced apoptosis in all lines.

Example 16. SAG expression protects cells from DNA fragmentation

The sensitivity of these SAG-transfected cells to OP-induced apoptosis was examined next by monitoring DNA fragmentation, a hallmark of apoptosis. Subconfluent (80-90%) SAG transfected cells expressing wildtype SAG, SAG mutant-1, SAG mutant-2, or vector control cells, were seeded at 3.5 x 10^6 per 100 mm dish and exposed after 16-24 hours to 150 μM OP, 125 μM zinc sulfate, or 200 μM H_2O_2 for 24 hours. Both detached and attached cells in 2 x 100 mm dishes were harvested and subjected to DNA fragmentation analysis as follows. Cells were collected by centrifugation and lysed with lysis buffer (5 mM Tris-HCL, pH 8; 20 mM EDTA; 0.5% Triton-X100) on ice for 45 minutes. Fragmented DNA in the supernatant of a 14,000 rpm centrifugation (45 minutes at 4°C) was extracted twice with phenol/chloroform and once with chloroform and precipitated by ethanol and salt. The DNA pellet was washed once with 70% ethanol and resuspended in TE buffer with 100 μg/mL RNase at 37°C for 2 hours. The fragmented DNA was separated in 1.8% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light.

OP induced apoptosis in the vector control cells. Less DNA fragmentation was observed in wild type SAG transfected cells compared to control cells. SAG mutant 1, which does not encode the zinc ring-finger motif, did not show any protection against OP-induced DNA fragmentation, whereas SAG mutant 2, which retains the zinc ring finger domain, still showed protection. These results suggest that overexpression of SAG protein protects cells against OP-induced apoptosis, and the zinc ring finger domain is required for this protective activity.

Since SAG protein contains a zinc ring finger motif, the sensitivity of SAG transfectants to zinc treatment was examined next. Zinc induced apoptosis in DLD-1 cells

transfected with the vector only. Induction of apoptosis was limited by SAG overexpression, which showed much less DNA fragmentation than the control lines. This data suggests that the SAG protein binds to and chelates zinc through the zinc ring finger domain and thus provides increased resistance to zinc toxicity compared to non-transfected cells.

Another feature of SAG is the formation of oligomers after exposure to H_2O_2. Cells may be protected from H_2O_2 induced toxicity by SAG oligomerization. SAG-transfected cells were, therefore, treated with H_2O_2 followed by assays for DNA fragmentation. H_2O_2 induced apoptosis in DLD-1 cells. SAG protein overexpression partially protected cells from H_2O_2-induced apoptosis, as evidenced by a reduction in DNA fragmentation. Taken together, these results demonstrate that SAG affords at least some protection against apoptosis induced by redox compounds such as OP and H_2O_2 and also against apoptosis caused by zinc.

Example 17. Antisense SAG expression inhibits tumor cell growth

To test the growth effects induced by SAG expression, DLD-1 cells were transfected with the neo control vector, or vectors expressing SAG, SAG mutants 1 or 2, or antisense SAG, as described above. Neomycin resistant colonies were selected with G418 (600 μg/mL) for 18 days and stained with 50% methanol/10% acetic acid/0.25% Coomassie Blue.

A stable DLD-1 transfectant expressing antisense SAG mRNA (D15-1) was cloned after G418 selection in order to examine potential changes in tumor cell phenotype caused by decreased SAG expression. Subconfluent D15-1 cells, along with the vector control cell (D1-6), and SAG (sense) overexpressing cells (D12-1 and D12-8) were metabolically labeled and subjected to immunoprecipitation using SAG protein antibody as described above. Densitometric quantitation of SAG protein expression using a computing densitometer, (Molecular Dynamics) was performed according to the manufacturer's instructions. The number was calculated by arbitrarily choosing the value from the vector control cell D1-6 as 1. Antisense SAG transfected cells (D15-1) exhibited a 60% reduction in endogenous SAG protein. Monolayer growth of DLD-1 cells was significantly inhibited by antisense SAG transfection. None of the other transfectants were growth-inhibited, as compared to the neo control.

It was next examined whether antisense SAG-transfected cells would exhibit growth inhibition in soft agar. D15-1 cells, along with transfectants expressing wildtype SAG (D12-8), SAG mutant-1 (D3-3), SAG mutant-2 (D4-2), as well as the neo control (D1-3)

were grown in 0.25% agar medium for 14 days. Colonies containing greater than 16 cells were counted. Three independent experiments, each run in duplicate, were performed. Shown is the mean +/- standard error of the mean. As shown in Figure 2, down-regulation of SAG in D15-1 cells did cause significant growth inhibition of DLD-1 cells as reflected by 75% reduction of soft agar colony number when compared to the neo control (D1-3), SAG (sense) expressing line, D12-8, and SAG mutants (D3-3, D4-2).

In a further study, 4 x 10^6 confluent D15-1 cells along with parental DLD-1 cells, the vector control D1-6, and SAG wildtype transfectant D12-1 cells were inoculated subcutaneously into SCID mice (Taconic Farms, Germantown, New York), 10 mice per group. Tumor growth was observed twice a week. The average tumor size/mass for 10 mice was plotted against time post injection up to 24 days. When implanted into SCID mice, antisense expressing line D15-1 failed to form tumors up to 24 days after inoculation, whereas substantial tumor growth was observed in parental DLD-1 cells, the neo control D1-6 cells, and SAG (sense) expressing D12-1 cells (Figure 3). All these experiments demonstrate that downregulation of SAG expression leads to growth inhibition of tumor cells, and further indicates that SAG is a cellular protective molecule.

Example 18. Cancer gene therapy using adenovirus expressing antisense SAG

Since antisense SAG expression has been shown to inhibit tumor growth both *in vitro* and *in vivo* (example 17), SAG can be used as a target for cancer gene therapy. Methods for conducting cancer gene therapy are well known in the art (see Zhang and Fang, Exp. Opin, Invest. Drugs 4: 487-514, 1995 and Zhang et al., Adv. Pharmacol. 32: 289-341, 1995).

Tumor cell lines with endogenous SAG expression, including, but not limited to DLD-1 (colon), Du145 (prostate), G401 (kidney), H2009 (lung) and HONET-1 (nasopharynx), are used to establish the tumor models,. Tumor cells from tissue culture are suspended in PBS at a concentration of 5 x 10^7/mL and stored on ice. 0.2 mL of the cell suspension (containing approximately ten million cells) is subcutaneously injected into the flank of 6- to 8-week-old athymic nude mice and tumors are allowed to grow for 30-40 days or until the average tumor size reaches 5 mm.

Recombinant adenoviral vectors expressing antisense human SAG, driven by the CMV promoter (Ad.CMV-SAG) were produced by co-transfecting a shuttle plasmid (pJM17, circularized Ad5 genome) and a recombinant plasmid (pEC-SAG; a CMV driven plasmid containing left arm of Ad5 genome) into 293 cells.

Tumors are injected with either 0.1 mL of recombinant adenoviral solution (1-5 x 10^{10} pfu/mL) or 0.1 mL of PBS alone as a control. Daily treatment is performed for 2 days and after 1 week without treatment, daily treatment is resumed for 3 days. The tumor size is measured daily for 2 weeks. To test combinatorial therapy with oxygen radical-generating reagents or irradiation, the treated group is subdivided into three sub-groups (10 mice per subgroup): group A receives adenovirus alone (see above); group B receives adenovirus and at the same time receives an intraperitoneal injection of adriamycin (3 mg/kg) an oxygen radical-generating reagent, and group C receives adenovirus plus irradiation at 350cGy of cesium-137. Some tumor-bearing mice will only receive the same dose of adriamycin or irradiation as drug or irradiation controls,.

Expression of antisense SAG blocks endogenous SAG synthesis, which renders tumor cells supersensitive to oxygen radicals. Significant tumor shrinkage in treated tumors with or without drugs or radiation, as compared with the vehicle control, indicates the efficacy of this therapy. The tumors in both control and treated groups can be further examined histologically. Samples can be immediately embedded in optimal cutting temperature compound (Miles, Inc. Elkhart, Indiana) and snap-frozen in liquid nitrogen for frozen section preparation (3-5 μm) for enzymatic staining (e.g., terminal deoxynucleotidyl transferase (Boehringer Manheim, Indianapolis, Indiana) staining for apoptosis) or immunohistochemical staining for expression of the antisense SAG. Alternatively, the samples may be fixed in 10% formalin for histologic sectioning and analyze with hematoxylin-eosin (Sigma, St. Louis, Missouri) staining.

Example 19. SAG functions as a oxygen radical scavenger to prevent oxygen radical induced damages

SAG protein contains 12 cysteine residues and forms disulfide bonds both intermolecularly and intramolecularly after exposure to hydrogen peroxide. SAG protein also binds to heme, which can modulate oxidants by oxidation/reduction of Fe(++). This oxidative buffering activity may qualify SAG as an oxygen radical scavenger.

Yeast cells having deletions in antioxidant enzyme genes [superoxide dismutase (SOD) and catalase (CAT)] are supersensitive to superoxide anion and hydrogen peroxide (Longo et al. (1997), J. Cell Biol. 137:1581-1588). Yeast cells that lack (a) Cu, Zn-SOD, (b) Mn-SOD, (c) both Cu, Zn-SOD and Mn-SOD, and (d) CAT have been transfected with human SAG expression plasmids. Sensitivity of these transfected cells to oxygen radical producing compounds such as paraquat (a superoxide anion generating compound) and

hydrogen peroxide are tested in yeast growth assays and compared to the growth of the same host cells transfected with vector controls. Rescue of these yeast cells from oxygen radical-induced cell killing indicates that SAG is an effective oxygen radical scavenger.

Example 20. Prevention of IL-1β induced brain injury during ischemia by SAG administration

It has been previously shown that middle cerebral artery occlusion in rats causes overexpression of interleukin-1 which induces brain injury by the release of free radicals (Yang et al., Brain Research 751:181-188, (1997)). Two experiments are conducted to test whether SAG, by scavenging free radicals released, will prevent brain damage.

In the first experiment, human SAG is subcloned into an adenovirus vector driven by RSV promoter (AdRSV-SAG). The adenoviral suspension is injected stereotactically into the lateral ventricle to ensure SAG expression in brain. Five days after administration of adenovirus, middle cerebral artery is occluded in animals for 24 hours as described (Yang et al., Brain Research 751:181-188, (1997)). Brain edema (as measured by brain water content) and cerebral infarct size, measured by histological techniques (Yang et al., Stroke 23:1331-1336, (1992)) is determined. As compared to the vector control, any reduction of brain edema and infarction size indicates SAG protection against free radical induced damage.

In the second experiment, middle cerebral artery occlusion is performed with the rat suture model, allowing either permanent (6 hours) or temporary occlusion (3 hours of occlusion and 3 hours of reperfusion) (Yang and Betz, Stroke, 25:1658-1665, (1994)). Rats then receive an injection of purified SAG protein at the size of occlusion. Brain water, ion contents, and infarct volume are measured to determine brain infarction and blood-brain barrier disruption. As compared to injection of the vehicle control, reduction in brain infarction size and blood-brain barrier disruption indicates a SAG protective effect.

Example 21. Human cancer diagnosis using SAG as a marker:

Two SAG deletion mutants in human cancer cell lines originating from colon and testis have been identitifed. Twelve pairs of colon carcinomas and adjacent normal tissues were collected from 12 patients. Genomic DNA and total RNA are isolated from these samples and subjected to PCR amplification. The resulting amplification products are analyzed for detection of SAG deletion mutations by methods well known in the art, including but not limited to RNA protection assays, DNA sequencing, hybridization, and gel

electrophoresis for deletion mutants. Mutations detected in tumor tissues but not in normal adjacent tissues indicate that they are tumor specific mutations and can be used as a diagnostic tool in the clinic for colon as well as testicular carcinomas.

Example 22: The yeast homolog of human SAG gene is essential for yeast growth

To further understand the function of SAG, yeast SAG knock-out mutants were constructed by homologous recombination. The construct used to knockout yeast SAG was made by PCR of a kanamycin cassette from kanMX4 plasmid (Wach et al., Yeast 10:1793-1808, 1994). The primers used for PCR were SAGKanMX4-5: 5′-TTCTCCAGTGGCAGAGAACTTTAAAGAGAAATAGTTCAAC CGTACGCTGCAGGTCGAC-3′ (SEQ ID 17), and SAGKanMX4-3: 5′-ACCTCGGTA TGATTTAAATGTTTACGGGCAATTCATTTTT ATCGATGAATTCGAGCTCG-3′ (SEQ ID 18). The primer SAGKanMX4-5 consists of yeast SAG DNA sequence (ATCC Accession number Z74876) immediately upstream of the initiation codon ATG (underlined) and the upstream kanamycin cassette sequence at its 3′-end. Primer SAGKanMX4-3 consists of yeast SAG DNA sequence immediately downstream of the stop codon TGA (underlined) and the downstream kanamycin cassette sequence at its 3′-end.

PCR was conducted for 5 cycles at 94°C 1 min, 50°C, 1.5 min, 72°C 2 min, followed by 25 cycles at 94°C, 1 min, 56°C, 1.5 min, 72°C 2 min, followed by a 10 min extension at 72°C. The resulting PCR product (1.5 kb) was gel-purified using Qiaex II gel-purification kit (Qiagen) according to the manufacturer's instruction, and was used to transfect the diploid yeast strain Y21 using the YEASTMAKER yeast Transformation System (ClonTech Laboratory, Inc.) according to the manufacturer's instruction. Following transfection, yeast cells were grown in YPD media (Difco) containing G418 (200 μg/mL, BRL) to select transfectants containing the kanamycin cassette, which have had the yeast SAG deleted by homologous recombination.

Several G418-resistant clones were selected and assayed to determine whether heterozygous or homozygous deletions had been produced. The primers used are SAGPCR-5: 5′-TTCTCCAGTGGCAGAGAAC-3′ (SEQ ID 19) and SAGPCR-3: 5′-ATGATTTAAATGTTTACGGGC-3′ (SEQ ID 20). These primers constitute fragments of SAGKanMX4-5 and SAGKanMX4-3, respectively, and flank the entire yeast SAG coding region. PCR of wildtype yeast SAG produces a 0.35 kb band, whereas PCR of SAG deletion

mutants give rise to 1.5kb band, consisting of the kanamycin cassette. Both the 0.35 kb and 1.5 kb fragments were generated in all of the clones tested, indicating that heterozygous mutants were produced. Identical knock-out experiments were conducted with haploid yeast cells (InvSC1 from In Vitrogen) and no G418-resistant clone was isolated.

The failure to isolate homozygous yeast SAG deletion mutants suggests that yeast SAG is essential for growth. To confirm this, 12 individual heterozygous yeast strains (y21-ySAG/ySAG::Kan) were sporulated to determine if yeast SAG-kan haploids were viable. The strains were inoculated into minimal potassium acetate sporulation media, supplemented with uracil, lysine, adenine and tryptophan (Kassir, and Simchen, G. Method Enzymol. 194, 94-110, 1991) and grown at 30°C for 7 days. Tetrads was dissected into 4 haploid offspring from each strain. For dissection, a clamp of cells from the sporulation plate was suspended in 100 µL of 1 M glycerol containing 0.5 mg/mL zymolase T20. After 30 min at 37°C, the suspension was diluted with 800 µL sterile water and put on ice. A loop of suspension was struck across a YPD plate and examined under a Zeiss Tetrad microscope for tetrads. The glass microneedle of the scope was used to dissect 4 tetrads from each strain. Two of these four haploid cell should contain wildtype SAG, while the other two should contain a yeast SAG deletion. In all 12 clones, only two out of four dissected cell grew, and none were viable in YPD medium supplemented with G418, indicating that viable cells did not contain the kanamycin cassette or the SAG deletion. The experiment clearly demonstrate that SAG is essential for yeast growth, further demonstrating its evolutionary importance.

To determine if *ySAG* is required for normal growth or simply for germination, *hSAG* was cloned into a yeast expression vector with *URA3* selectable marker. The *hSAG-URA* plasmid was then transformed into heterozygous ySAG knockout cells, and transformants were selected on URA-minus plates. Clones expressing hSAG (measured by Western blot analysis) were sporulated and tetrads were dissected. Viable colonies were then screened on either YPD alone, or YPD+G418, or YPD+5-fluoroorotic acid (5-FOA; used to select against the URA3-containing centromere plasmid (Boeke et al., Mol. Gen. Genet, 1984;197:345). Again the *hSAG-URA3* plasmid complemented the *ySAG::kan* allele, as all four haploids from four individual tetrads grew. When grown on YPD+G418 plates, two haploids from each tetrad die, indicating that they contain the wildtype *ySAG* gene. Other two haploids from each tetrads survived, indicating they contained *ySAG::kan* allele. When these latter colonies were grown on YPD+5-FOA plates, which selects against URA3 plasmid, all failed

to grow, indicating that *ySAG* is essential for normal vegetative growth and not simply for sporulation.

Example 23: Human SAG rescue of yeast SAG knockout phenotype

To examine whether human SAG can rescue death phenotype of yeast SAG knockout, wildtype human SAG, along with the SAG mutants (MM3, sequence ID 25; MM10, sequence ID 39; and MM14, sequence ID 47, Figure 1A) were constructed into a plasmid with Trp selection marker and transfected into heterozygous yeast strain (y21-SAG/ySAG::Kan) as described above. The clones grown in Trp-minus/G418-plus plates were examined by Western blot analysis for SAG expression. The clones expressing human SAG were sporulated and dissected. In 10 wildtype human SAG clones, 3 or 4 haploids are viable. Some of them contain yeast SAG, whereas the others contain ySAG K/O plus human SAG, indicating human wildtype SAG can complement yeast SAG knockout. All three mutant clones (total of 41 tested) gave rise to 1 or 2 haploids and all survival haploids contains yeast SAG, indicating that human SAG mutants cannot complement yeast SAG knockout.

Example 24: SAG binds to metals

Since SAG contains a zinc-ring finger domain, it has the potential to bind with metals. To measure potential metal binding of SAG, electrospray ionization mass spectrometry (ESI-MS) (Fenn et al., 1989) was used to compare the molecular mass of SAG under denaturing and non-denaturing solution conditions (Loo, 1997; Witkowska et al., 1995).

ESI-MS was performed with a double focusing hybrid mass spectrometer (Finnigan MAT 900Q, Bremen, Germany) with a mass-to-charge (m/z) range of 10,000 at 5 kV full acceleration potential. A position-and-time-resolved-ion-counting (PATRIC) scanning array detector was used. An ESI interface based on a heated metal capillary inlet and a low flow micro-EsI source (150 nL/min analyte flowrate) were used (Sannes-Lowery et al., 1997). The metal capillary temperature was maintained around 150-200°C for metal-protein complex studies. Recombinant protein under 7 M urea-denaturing solution was refolded by dialyzing in 50 μM $ZnCl_2$ for 3 days with three changes of buffer. Prior to ESI-MS measurement, the SAG solution was washed with a solution of 10 mM ammonium bicarbonate (pH 7) and 1 mM DTT, and excess zinc was removed by centrifugal ultrafiltration by passing through a 10 kDa molecular weight cut-off centrifugal filtration cartridge (Microcon-10 microconcentrator, Amicon, Beverly, MA). For the ESI-MS

analysis, a small portion of the filtered SAG protein solution was diluted into either a denaturing solvent (80:15:5 acetonitrile:water:acetic acid v/v/v, pH 2.5) or a non-denaturing solution (10 mM ammonium bicarbonate and 1 mM DTT, pH 7).

Zinc binding of SAG was first measured. Under a denaturing acidic solution (pH 2.5 and high organic concentration) where the protein is not expected to retain metal-binding characteristics even in the presence of zinc, the molecular mass of SAG was measured to be 12550, in close agreement with the expected mass for the apo-protein (12552 Da). The ESI-MS analysis of the SAG protein in a non-denaturing aqueous solution (pH 7) resulted in an increase in mass to 12733 and 12800 Da. These masses are consistent for the holo-protein binding 3 and 4 zinc metal ions, respectively.

Copper binding to SAG was also measured. As little as 1 μM $CuSO_4$ in the dialysis solution causes SAG precipitation with a blue (copper) color, suggesting a copper binding. Next, using ESI-MS, the potential copper binding of SAG was measured in a non-denaturing solution described above. Addition of copper acetate to a final concentration of 10 μM resulted in a further inccrease in mass to approximately 12929 Da. However, a precise mass could not be obtained, as a wide distribution of copper adducts appears to bind to SAG protein. Adding copper to higher concentrations resulted in precipitation of the protein.

Example 25: SAG minimizes or prevents LDL oxidation induced by copper ion or a free radical generator

Due to its H_2O_2 buffering and metal binding, it was reasoned that SAG may prevent oxidation of macromolecules induced by metal or free radical generator. An LDL (low density lipoprotein) oxidation induced by copper ion or a free radical generator, AAPH (2,2-azobis-2-amidinopropane hydrochloride), was used as a model to test potential protection activity of SAG against lipid peroxidation.

Lipoproteins (100 μg of protein/mL, Intraocel) were incubated with 10 μM $CuSO_4$ or with 5 mM AAPH for 4 hours at 37°C in the presence of various concentrations of purified SAG protein. AAPH is a water-soluble azo compound that thermally decomposes and generates water soluble peroxyl radicals at a constant rate (Frei et al., 1988). Oxidation was terminated by the addition of 10 μM butylated hydrozytoluence (BHT) and refrigeration at 4°C. The extent of lipoprotein oxidation was measured by the TBARS assay, using malondialdehyde (MDA) for the standard curve, as described (Buege & Aust, 1978).

Copper-induced LDL oxidation, as measured by the formation of thio barbituric acid reactive substances (TBARS), was slightly enhanced by SAG at low concentrations. At higher SAG concentrations, however, a dose-dependent inhibition (up to 90%) of LDL oxidation was observed. Inhibition was heat-resistant since heat-treated (60°C for 15 min) SAG still retains the activity, suggesting that enzymatic activity is not involved. Inhibitory activity was, however, completely or partially abolished by pretreatment of SAG with alkylating reagents NEM and p-hydroxy mercury benzoate (PHMB), respectively. The results indicated that free SH groups in SAG are the major contributors to this activity. Furthermore, metallothionein, a small metal binding protein consisting of 20 cysteine residues out of 61 amino acids (Nordberg & Kojima, 1979) showed a similar inhibitory curve as SAG. Glutathion (GSH), an additional cysteine containing peptide showed a 25% inhibition at a concentration of 100 μM. Inhibition of copper-induced LDL oxidation was, however, not observed in other known antioxidant enzymes such as superoxide dismutase, catalase or other proteins such as BSA, and cytochrome C. These results clearly showed that by binding and chelating copper ion through its free SH groups, SAG prevents copper-initiated free radical reactions leading to LDL oxidation and superoxide or hydrogen peroxide appear not to be involved in the process. To test whether SAG protection against LDL oxidation was solely mediated through copper binding, we initiated LDL oxidation by AAPH, a free radical generator. In this metal-ion free system, SAG also protects LDL oxidation (up to 85%) at a concentration of 59 μM (750 μg/mL). Thus, by metal binding and free radical scavenging, SAG acts as a protector against lipid peroxidation.

Example 26. SAG protects cytochrome C release and caspase activation induced by metal ions

Since cytochrome C release from mitochondria and caspase activation are the key events in apoptosis (Liu et al., 1996; Yang et al., 1997; Li et al., 1997; Hengartner, 1998, for review, see Mignotte & Vayssiere, 1998), the levels of cytochrome C released into cytoplasm and potential activation of caspase upon metal treatments were measured. Treatment of cells with $ZnSO_4$ induces a time-dependent release of cytochrome C in cytoplasm. Compared to the vector control cell (D1-6), the SAG overexpressing cell (D12-1) has much less cytoplasmic release of cytochrome C. Likewise, activation of caspase 7, shown as disappearance of pro-enzyme form, was seen in a time-dependent manner post zinc treatment. More activation was seen in vector control cell (D1-6) than that in the SAG overexpressing

cell (D12-1). A similar result was obtained with CPP32 (caspase 3) activation. A significant difference, however, was not seen in cytochrome C release or caspase activation between D1-6 and D12-1 cells upon copper treatment. This is consistent with the lack of difference in morphological changes between the two lines upon copper treatment, although DNA fragmentation was obvious only in the vector control cells. To further examine potential protection of SAG against metal-induced cytochrome C release and CPP32 activation, cytochrome C release and CPP32 activation was measured in 293 cells transiently transfected with SAG expressing plasmid followed by exposure to copper. A significant amount of cytochrome C started to release 6 hours post $CuSO_4$ (2.0 mM) treatment and lasted up to 12 hours. Expression of SAG delayed cytochrome C release for up to 16 hours. Activation of caspase 7 was seen in the vector control cells 12 hours and 16 hours post copper treatment. No significant activation was seen in SAG transfectants. The similar result was seen with CPP32 antibody. For zinc treatment, no difference was detected in cytochrome C release and caspase activation between control cells and SAG transfectants, consistent with the lack of difference in morphological signs of apoptosis. These results indicate that metal treatment induces cytochrome C release and caspase activation during apoptosis which can be largely prevented or delayed by SAG and there is a good correlation between morphological signs of apoptosis and cytochrome C release/caspase activation.

Example 27: SAG protects against neuronal apoptosis

SAG was transfected into HY5Y human neuroblastoma cells and a few stable lines were selected which expressed exogenous SAG as determined by Western blot. One SAG-transfectant (SYW-20) and a vector control (SYV-3) were used to determine their sensitivity to metal ions, zinc and copper. Treatment with 1.25 mM $CuSO_4$ or 200 μM $ZnSO_4$ for 16 hours induced cell shrinkage and detachment in the neo control cells, but to a less extent in SAG-expressing cells. The morphological difference was more obviously seen with the zinc treatment. To determine the nature of cell death, we performed TUNEL assay, a fluorescein labelling assay of free 3′-OH termini generated from cleavage of genomic DNA during apoptosis.

In Situ cell death assay (TUNEL assay) was performed according to the manufacturer's instructions (Boehringer Mannheim). Briefly, 5×10^4 cells were plated into the 8-well glass slides. After treatment with 1.25 mM copper ($CuSO_4$) or 200 μM zinc ($ZnSO_4$) for 16 hours, cells were fixed with 0.5% glutaraldehyde for 10 min, then washed

with PBS twice. The fixed cells were incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. The TUNEL reaction mixture (50 µL) was added to samples and incubated for 1 hour at 37°C followed by 3 times wash with PBS. Samples were embedded with antifade prior to analysis under a fluorescence microscope. Substantially more fluorescein staining was seen in the vector control cells after 16 hours treatment with 1.25 mM $CuSO_4$, or 200 µM $ZnSO_4$. The results indicate that expression of SAG protects neuronal cells from apoptosis.

Example 28. SAG stimulates proliferation

To test potential growth stimulation activity, SAG RNA (8 µg/mL or 25 µg/mL), along with the control β-galactosidase (25 µg/mL), was injected into serum-starved NIH 3T3 fibroblast monolayer. Approximately 50 cells attached to the glass coverslip within an etched circle were injected. A 3-hour pulse of [^{3}H]thymidine (5 µCi/mL, Amersham) was performed 10 to 24 hours after injection. Cultures were washed with isotonic phosphate-buffered saline and fixed in 3.7% (vol/vol) formaldehyde. Induction of [^{3}H]thymidine incorporation (an indicator of DNA synthesis) into the nuclei of serum-starved fibroblast cells was obviously observed in SAG-injected cells. In contrast, injection of β-galactosidase does not induce DNA synthesis and no [^{3}H]thymidine incorporation was observed. The results clearly indicate that human SAG has proliferative activity to stimulate cell growth.

Growth promotion activity of SAG was also examined in human neuroblastoma cells (SY5Y), overexpressing hSAG protein by hSAG cDNA transfection. Both the vector-expressing control cells and SAG overexpressing cells were first serum-starved for 48 hours, followed by 3H-thymidine labelling for 16 hours in either serum-starved or 1% serum conditions. Cells were washed, lysed and counted in a liquid scintillation counter for 3H, an assay for the measurement of 3H-thymidine incorporation into DNA (S-phase entry). Compared to the vector control cells, SAG-expressing cells have 10-fold more 3H-thymidine incorporation in both conditions (serum-free or 1% serum), indicating that SAG stimulates cell proliferation/growth.

Growth promotion activity of SAG was also examined in yeast. As described in Example 22, the yeast homolog of human SAG gene is essential for yeast growth. To correlate yeast growth rate with SAG expression, hSAG expressing plasmid was constructed under control of Gal promoter. The plasmid was transformed into heterozygous ySAG knockout and transformants were sporulated and dissected. Haploid ySAG knockout clone

that contained hSAG plasmid was identified and analyzed. In the uninduced condition, little SAG expression due to the leakness of the promoter led to formation a tiny clone compared to the full size wildtype clone. Under induced condition, SAG expression level increased and clone size also increased. This experiment clearly demonstrated that SAG promotes cell growth in a dose-dependent manner.

It is to be understood that the invention is not to be limited to the exact details of operation, or to the exact compounds, compositions, methods, procedures or embodiments shown and described, as obvious modifications and equivalents will be apparent to one skilled in the art, and the invention is therefore to be limited only by the full scope of the appended claims.

I claim

1. An isolated and purified DNA sequence substantially similar to the DNA sequence shown in SEQ ID 1.

2. An isolated and purified DNA sequence that hybridizes to the DNA sequence shown in SEQ ID 1 under high stringency hybridization conditions.

3. An isolated and purified DNA sequence that consists essentially of the DNA sequence shown in SEQ ID 1.

4. A recombinant DNA molecule comprising the isolated and purified DNA sequence of Claim 1, 2, or 3 subcloned into an extra-chromosomal vector.

5. A recombinant host cell comprising a host cell transfected with the recombinant DNA molecule of Claim 4.

6. A recombinant host cell deposited with the ATCC under accession number 98402.

7. An isolated and purified DNA sequence substantially similar to the DNA sequence shown in SEQ ID 3.

8. An isolated and purified DNA sequence that hybridizes to the DNA sequence shown in SEQ ID 3 under high stringency hybridization conditions.

9. An isolated and purified DNA sequence that consists essentially of the DNA sequence shown in SEQ ID 3.

10. A recombinant DNA molecule comprising the isolated and purified DNA sequence of Claim 7, 8, or 9 subcloned into an extra-chromosomal vector.

11. A recombinant host cell comprising a host cell transfected with the recombinant DNA molecule of Claim 10.

12. A recombinant host cell deposited with the ATCC under accession number 98403.

13. A recombinant host cell deposited with the ATCC under accession number 98404.

14. A recombinant host cell deposited with the ATCC under accession number 98405.

15. An isolated and purified DNA sequence selected from the group consisting of SEQ ID 11, SEQ ID 13, SEQ ID 21, SEQ ID 23, SEQ ID 25, SEQ ID 27, SEQ ID 29, SEQ ID 31, SEQ ID 33, SEQ ID 35, SEQ ID 37, SEQ ID 39, SEQ ID 41, SEQ ID 43, SEQ ID 45, SEQ ID 47, and SEQ ID 49.

16. A recombinant DNA molecule comprising an isolated and purified DNA sequence of Claim 15, subcloned into an extra-chromosomal vector.

17. A recombinant host cell comprising a host cell transfected with a recombinant DNA molecule of Claim 16.

18. A substantially purified recombinant polypeptide, wherein the amino acid sequence of

the substantially purified recombinant polypeptide is substantially similar to the amino acid sequence shown in SEQ ID 2.

19. A substantially purified recombinant polypeptide, wherein the amino acid sequence of the substantially purified recombinant polypeptide consists essentially of the amino acid sequence shown in SEQ ID 2.

20. A substantially purified recombinant polypeptide, wherein the amino acid sequence of the substantially purified recombinant polypeptide is substantially similar to the amino acid sequence shown in SEQ ID 4.

21. A substantially purified recombinant polypeptide, wherein the amino acid sequence of the substantially purified recombinant polypeptide consists essentially of the amino acid sequence shown in SEQ ID 4.

22. A substantially purified recombinant polypeptide, wherein the amino acid sequence of the polypeptide is selected from the group consisting of SEQ ID 12, SEQ ID 14, SEQ ID 22, SEQ ID 24, SEQ ID 26, SEQ ID 28, SEQ ID 30, SEQ ID 32, SEQ ID 34, SEQ ID 36, SEQ ID 38, SEQ ID 40, SEQ ID 42, SEQ ID 44, SEQ ID 46, SEQ ID 48, and SEQ ID 50.

23. An antibody that selectively binds polypeptides with an amino acid sequence substantially similar to the amino acid sequence of Claim 18, 19, 20, 21 or 22.

24. A method of detecting SAG protein in cells, comprising contacting cells with the antibody of Claim 23 and incubating the cells in a manner that allows for detection of the SAG protein-antibody complex.

25. A diagnostic assay for detecting cells containing SAG mutations, comprising isolating total genomic DNA from the cell and subjecting the genomic DNA to PCR amplification using primers derived from the isolated and purified DNA sequence of Claim 1, 2, 3, 7, 8, 9, or 15, and determining whether the resulting PCR product contains a mutation.

26. A diagnostic assay for detecting cells containing SAG mutations, comprising isolating total cell RNA, subjecting the RNA to reverse transcription-PCR amplification using primers derived from the isolated and purified DNA sequence of Claim 1, 2, 3, 7, 8, 9, or 15 and determining whether the resulting PCR product contains a mutation.

27. A method of isolating RNA containing stretches of polyA or polyC residues, comprising

(a) contacting an RNA sample with SAG protein in RNA binding buffer in the presence of a reducing agent;

(b) incubating the RNA-SAG protein mixture with the antibody of Claim 23;

(c) isolating the antibody-SAG protein-RNA complexes; and

(d) purifying the RNA away from the antibody-SAG protein complex.

28. A method of isolating RNA containing stretches of polyU residues, comprising

(a) contacting an RNA sample with SAG protein in RNA binding buffer in the absence of reducing agents;

(b) incubating the RNA-SAG protein mixture with the antibody of Claim 23;

(c) isolating the antibody-SAG protein-RNA complexes; and

(d) purifying the RNA away from the antibody-SAG protein complex.

29. A method for isolating genes induced during cell apoptosis, comprising:

(a) treating one set of cells with OP and not treating a control set of cells;

(b) isolating RNA from each set of cells;

(c) subjecting the RNA from each set of cells to the differential display procedure, wherein the RNA is reverse transcribed into cDNA and the cDNA is subjected to the polymerase chain reaction;

(d) identifying cDNAs that are expressed in the OP-treated set of cells and not in the control set of cells; and

(e) cloning the OP-induced cDNAs.

30. A method for protecting cells from apoptosis induced by redox reagents, comprising introducing into the cells an expression vector comprising the isolated and purified DNA sequence of Claim 1, 2, 3, 7, 8, 9, or 15, which is operatively linked to a DNA sequence that promotes the high level expression of the isolated and purified DNA sequence in the cells.

31. A method for inhibiting the growth of tumor cells, comprising introducing into the tumor cells an expression vector comprising the isolated and purified DNA sequence of Claim 1, 2, 3, 7, 8, 9, or 15, which is operatively linked to a DNA sequence that promotes the high level expression of the antisense strand of the isolated and purified DNA sequence in the cells.

32. A method for purifying SAG protein from bacterial cells comprising:

a) transfecting a bacterial host cell with a vector comprising the isolated and purified DNA sequence of Claim 1, 2, 3, 7, 8, 9, or 15 operatively linked to a promoter capable of directing gene expression in a bacterial host cell;

b) inducing expression of the isolated and purified DNA sequence in the bacterial cells;

c) lysing the bacterial cells;

d) isolating bacterial inclusion bodies;

e) purifying SAG protein from the isolated inclusion bodies.

33. A pharmaceutical composition comprising the substantially purified recombinant polypeptide of Claim 18, 19, 20, 21, or 22 and a pharmaceutically acceptable carrier.

34. The pharmaceutical composition of Claim 33 wherein the substantially purified recombinant polypeptide comprises an oligomer.

35. A method of oxygen radical scavenging in an organism comprising administering an oxygen radical -reducing amount of the pharmaceutical composition of Claim 33 or 34 to the organism.

36. A method of promoting the healing of a wound comprising administering the DNA sequence of Claim 1 to cells associated with the wound.

37. A method of promoting or inhibiting the growth of plant cells comprising administering the DNA sequence of Claim 1 or a DNA sequence which is complementary to the DNA sequence of Claim 1 to plant cells.